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(57) Abstract

Multidrug resistant strains of *Mycobacterium tuberculosis* represent a considerable threat to public health worldwide. Resistance to isoniazid (INH), rifampicin or analogues thereof, or streptomycin, i.e. key components of anti-tuberculosis regimens, need frequently to be detected. The invention involves the detection of a mutation in either the *katG* gene (isoniazid resistance), the *rpoB* gene (rifampicin resistance) or *rpsL* gene (streptomycin resistance).

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RAPID DETECTION OF ANTIBIOTIC RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS

This invention relates to the rapid detection of strains of Mycobacterium tuberculosis that are resistant to antibiotics, particularly isoniazid, rifampicin and streptomycin. More particularly, this invention relates to a method of detecting antibiotic resistance in Mycobacterium tuberculosis, e.g. either as a result of mutations in the relevant genes or by nucleic acid hybridization. This invention also relates to a nucleic acid probe and a kit for carrying out the nucleic acid hybridization. The invention further relates to the chromosomal location of the katG gene and its nucleotide sequence.

BACKGROUND OF THE INVENTION

Despite more than a century of research since the discovery of Mycobacterium tuberculosis, the aetiological agent of tuberculosis, by Robert Koch, this disease remains one of the major causes of human morbidity and mortality. There are an estimated 3 million deaths annually attributable to tuberculosis (Snider, 1989), and although the majority of these are in developing countries, the disease is assuming renewed importance in the West due to the increasing number of homeless people and the impact of the AIDS epidemic (Chaisson et al., 1987; Snider and Roper, 1992).

Isonicotinic acid hydrazide or isoniazid (INH) has been used in the treatment of tuberculosis for the last forty years due to its exquisite potency against the members of the "tuberculosis" groups - Mycobacterium tuberculosis, M. bovis and M. africanum (Middlebrook, 1952; Youatt, 1969). Neither the precise target of the drug, nor its

mode of action, are known, and INH treatment results in the perturbation of several metabolic pathways. There is substantial evidence indicating that INH may act as an antimetabolite of NAD and pyridoxal phosphate (Bekierkunst and Bricker, 1967; Sriprakash and Ramakrishnan, 1970; Winder and Collins, 1968, 1969, 1970), and other data indicating that the drug blocks the synthesis of the mycolic acids, which are responsible for the acid-fast character of mycobacterial cell walls (Winder and Collins 1970; Quemard et al., 1991). Shortly after its introduction, INH-resistant isolates of Mycobacterium tuberculosis emerged and, on characterization, were often found to have lost catalase-peroxidase activity and to show reduced virulence in guinea pigs (Middlebrook et al., 1954; Kubica et al., 1968; Sriprakash and Ramakrishnan, 1970).

Very recently, INH-resistance has acquired new significance owing to a tuberculosis epidemic in the USA due to multidrug resistant (MDR) variants of M. tuberculosis (CDC, 1990; 1991a, b) and the demonstration that such strains were responsible for extensive nosocomial infections of HIV-infected individuals and health care workers (Snider and Roper, 1992). In view of the gravity of this problem, there exists a need in the art to determine the relationship between INH-resistance and catalase-peroxidase production.

More particularly, there is a need in the art to understand the molecular mechanisms involved in drug sensitivity. In addition, there is a need in the art to develop a simple test permitting the rapid identification of INH-resistant strains. Further, there is a need in the art for reagents to carry out such a test.

Rifampicin too is a majeur antibiotic used for the treatment of infections by mycobacterium, particularly Mycobacterium tuberculosis and Mycobacterium leprae. Because some mycobactéria grow slowly, possible rapid and efficient tests for the testing of resistance to rifampicin or analogues thereof must be made available. Likewise the invention aims at a rapid detection of strands of Mybobacterium tuberculosis which are resistant to streptomycin. Because of the development of resistance to streptomycin, the latter antibiotic has been used together with other antibiobics, e.g. isoniazid. Thus adequat treatment of tuberculosis should be preceded by rapid and efficient detection of resistances to the three majeur antibiotics, isoniazid, rifampicin and streptomycin.

SUMMARY OF THE INVENTION

Accordingly, this invention aids in fulfilling these needs in the art by providing a process for detecting in vitro the presence of cells of a Mycobacterium tuberculosis resistant to isoniazid and other drugs, such as rifampicin or analogues thereof, and streptomycin.

By analogues of rifampicin, a particularly meant derivatives of 3-formyl-rifamycin, particularly as a result of substitution the
rein for the sustituant present either in the naphtofuranonyl group or of the site chain at position 7 of the naphtofuranonyl group, or by the introduction or removal of a double band in the lateral chain.

In accordance with the invention, the detection of a resistance to isoniazid involves the detection of one or several litations in the katG gene of Mycobacterium

tuberculosis, particularly with respect to the nucleotide sequence of that same katG gene in mycobacterium tuberculosis that are not resistant to isoniazid.

Another process alternative for detecting in vitro the presence of nucleic acids of a Mycobacterium tuberculosis resistant to isoniazid, wherein the process comprises the steps of:

- contacting said nucleic acids previously made accessible to a probe if required under conditions permitting hybridization;
- detecting any probe that had hybridized to said nucleic acids;

wherein said probe comprises a nucleic acid sequence, which is 2.5 kb EcoRV-KpnI fragment of plasmid pYZ56 or of part thereof, and wherein said fragment contains a BamHI cleavage site, wherein said part is nonetheless sufficiently long to provide for the selectivity of the in vitro detection of a Mycobacterium tuberculosis resistant to isoniazid.

For instance, this process alternative comprises the steps of :

(A) depositing and fixing nucleic acids of the cells on a solid support, so as to make the nucleic acids accessible to a probe;

(B) contacting the fixed nucleic acids from step (A) with a probe under conditions permitting hybridization;

(C) washing the filter resulting from step (B), so as to eliminate any non-hybridized probe; and then

(D) detecting any hybridized probe on the washed filter resulting from step (C).

The probe comprises a nucleic acid sequence which is

present in a 2.5 kb EcoRV-KpnI fragment of plasmid pYZ56, wherein said fragment contains a BamHI cleavage site. This fragment has been found to be associated with intracellular DNA of isoniazid-sensitive Mycobacterium tuberculosis and is capable of distinguishing such antibiotic sensitive microorganisms from isoniazid-resistant Mycobacterium tuberculosis, which do not contain DNA that hybridizes with this fragment under the conditions described hereinafter.

This invention further provides nucleotide sequences, such as RNA and DNA, of isoniazid-resistant Mycobacterium tuberculosis encoding the region of the katG gene of Mycobacterium tuberculosis that imparts isoniazid sensitivity absent from isoniazid-resistant cells.

This invention also provides a probe consisting of a label, such as a radionuclide, bonded to a nucleotide sequence of the invention.

In addition, this invention provides a hybrid duplex molecule consisting essentially of a nucleotide sequence of the invention hydrogen bonded to a nucleotide sequence of complementary base sequence, such as DNA or RNA.

Also, this invention provides a process for selecting a nucleotide sequence coding for a catalase-peroxidase gene of Mycobacterium tuberculosis, or for a portion of such a nucleotide sequence, from a group of nucleotide sequences, which comprises the step of determining which of the nucleotide sequences hybridizes to a nucleotide sequence of the invention. The nucleotide sequence can be a DNA sequence or an RNA sequence. The process can include the step of detecting a label on the nucleotide sequence.

Further, this invention provides a kit for the

detection of Mycobacterium tuberculosis resistant to isoniazid. The kit comprises a container means containing a probe comprising a nucleic acid sequence, which is a 2.5 kb EcoRV-KpnI fragment of plasmid pYZ56, wherein the fragment contains a BamHI cleavage site. The kit also includes a container means containing a control preparation of nucleic acid.

The invention also covers compounds obtained as products of the action of the enzyme catalase, or a similar enzyme on isoniazid. The katG gene or a derivative of this gene which retains a similar activity can be used as a source of catalase protein. The new compounds are selected by reactivity on INH-resistant-mycobacterial strains by the antibiogram method such as described in H. David et al.'s "Methodes de laboratoire pour Mycobacteriologie clinique" edited by Pasteur Institute, ISBN N° 0995-2454.

BRIEF DESCRIPTION OF THE DRAWINGS

This invention will be described in greater detail by reference to the drawings in which:

Fig 1. shows the INH-resistant M. smegmatis strain, BH1 (Gayathri et al., 1975) (a derivative of strain MC^2-155) was transformed with a pool of M. tuberculosis-H37Rv shuttle cosmids (kindly provided by Dr. W.R. Jacobs, New York) and individual clones were scored for INH-susceptibility. Cosmid pBH4 consistently conferred drug susceptibility and the transformant overproduced catalase (assayed as in Heym, 1992). The restriction map of the DNA insert from pBH4 is shown along with that of the insert from pYZ55 - a plasmid containing katG of M. tuberculosis H37Rv, isolated on the basis of hybridization with an oligonucleotide probe

(5'-TTCATCCGCATGGCCTGGCACGGCGCGGGCACCTACCGC-3') designed to match the amino acid sequence from a conserved region of E. coli hydroperoxidase I (HPI). Restriction sites for the following enzymes are indicated : B, BamHl; C, Clal; E, EcoRV; H, HindIII, K, Kpnl; M, Smal; N, Notl; R, EcoRl; S, Sacl. Transformation of BH1 with a mycobacterial shuttle plasmid, pBAK14, Zhang et al., 1991, containing the 4.5 kb insert from pYZ55 similarly conferred INH-susceptibility. MIC's are also shown for BH1 transformed with subfragments derived from pYZ55 and inserted into pBAK14 in one (+) or other (-) orientation. The katG gene and the ability to confer INH-susceptibility both mapped to a 2.9 kb EcoRV-Kpnl fragment (pBAK-KE+).

Fig. 2 shows extracts from M. tuberculosis H37Rv and from E. coli strains transformed with a variety of plasmid constructs that were prepared for activity gel analysis as described previously (Zhang et al., 1991). Non-denaturing gels containing 8% polyacrylamide were stained for catalase (panel A) and peroxidase (panel B) activities as described by Wayne and Diaz (Wayne et al., 1986). Lane 1, M. tuberculosis H37Rv; 2, E. coli UM2 (katE, katG; 3, E. coli UM2/pYZ55; 4, E. coli UM2/pYZ56 (the 2.9 kb EcoRV-Kpnl fragment in pUC19, corresponding to pBAK-KE+ in Fig. 1); 5, E. coli UM2/pYZ57 (pYZ55 with a BamHl-Kpnl deletion, corresponding to pBAK-KB+ in Fig. 1). M. tuberculosis catalase and peroxidase activities migrated as two bands under these conditions (lane 1); the same pattern was seen for the recombinant enzyme expressed by pYZ55 (lane 3). pYZ56 (lane 4) expresses a protein of increased molecular weight due to a fusion between katG and lacZ' from the vector as shown in panel

C. Panel C also shows partial sequence alignment with E. coli HPI.

Fig. 3 shows an E. coli strain with mutations in both katG and katE (UM2 Mulvey et al., 1988) that was transformed with pUC19 vector alone, pYZ55 expressing M. tuberculosis katG and pYZ56 with high level expression of M. tuberculosis katG. Overnight cultures in Luria-Bertani broth supplemented with appropriate antibiotics were plated out in the presence of varying concentrations of INH and colony forming units were assessed. Results of a representative experiment are shown with error bars indicating the standard deviation observed in triplicate samples. Overexpression of M. tuberculosis katG similarly conferred susceptibility to high concentrations of INH in E. coli UM255 (katG, katE, Mulvey et al., 1988), but had no effect on catalase-positive strains such as E. coli TG1. In some experiments, high concentrations of INH had detectable inhibitory effect on growth of UM2 and UM255, alone, but in all experiments inhibition of pYZ56-transformants was at least 10-100 fold greater than that observed in the corresponding vector controls.

Fig. 4 shows Southern blots prepared using genomic DNA from different M. tuberculosis strains, digested with Kpnl, that were probed with (A) katG (the 4.5 kb Kpnl fragment), and (B) the SOD gene (1.1 kb EcoR1-Kpnl fragment, Zhang et al., 1991). Labelling of probes and processing of blots was performed as described previously (Eiglmeier et al., 1991; Maniatis et al., 1989). Lane 1, H37Rv; 2, strain 12 - MIC 1.6 μg/ml INH; 3, B1453 - MIC > 50 μg/ml INH (Jackett et al., 1978); 4, strain 24 - MIC > 50 μg/ml INH; 5, 79112 - INH-sensitive (Mitchison et

al., 1963); 6, 12646 - INH-sensitive (Mitchison et al., 1963); 7, 79665 - INH-sensitive (Mitchinson et al., 1963). INH susceptibilities were confirmed by inoculation of Lowenstein-Jensen slopes containing differing concentrations of INH.

Fig. 5. Organization of the katG locus. The upper bar corresponds to a stretch of the M. tuberculosis chromosome spanning the katG region and the positions of individual cosmids used to construct the map are shown below together with the original shuttle cosmid pBH4 and pYZ55. The locations of some key restriction sites (B, BamHI; K, KpnI) are shown together with the approximate location of the known genetic markers: fbpB encoding the alpha or 85-B antigen (Matsuo et al., 1988); katG, catalase-peroxidase; LL105, an anonymous λgt11 clone kindly supplied by Å Andersen; MPTR, major polymorphic tandem repeat (Hermans et al., 1992).

Fig. 6. A. Nucleotide sequence of the KpnI fragment bearing katG. This sequence has been deposited in the EMBL data-library under accession number X68081. The deduced protein sequence is shown in the one letter code. B. Alignment of the two copies of the 700 bp direct repeat with identities shown as * and - denoting pads introduced to optimize the alignment. Numbering refers to the positions in Fig. 2A.

Fig. 7. Distribution of katG in mycobacteria. A. Samples of different bacterial DNAs (1.5 μg) were digested with RsrII, separated by agarose gel electrophoresis and stained with ethidium bromide; lanes 1 and 7, size markers; M. leprae; lane 3, M. tuberculosis H37Rv; lane 4, M. gordonae; lane 5, M. szulgai; lane 6, M. avium. B. Hybridization of the gel in A, after

Southern blotting, with a katG specific probe.

Fig. 8. Primary structure alignment of catalase-peroxidases. The sequences are from M. tuberculosis H37RV, mtkatg; E. coli, eckatg (Triggs-Raine et al., 1988); S. typhimurium, stkatg; B. stearothermophilus, bspera (Loprasert et al., 1988) and yeast cytochrome c peroxidase (ccp; Finzel et al., 1984). The alignment was generated using PILEUP and PRETTY (Devereux et al., 1984)

and . denote gaps introduced to maximize the homology. Key residues from the active site and the peroxidase motifs (Welinder, 1991), discussed in the text, are indicated below the consensus.

Fig. 9. Western blot analysis of M. tuberculosis KatG produced in different bacteria. Proteins were separated by SDS-polyacrylamide gel electrophoresis then subjected to immunoblotting, and detection with antiserum raised against BCG, as described in Zhang et al., 1991.

Lane 1, soluble extract of M. tuberculosis H37Rv; lane 2, M. smegmatis MC^2155 harboring the vector pBAK14; lane 3, MC^2155 harboring pBAK-KK (katG+); lane 4, E. coli UM2 (katE, katG), lane 5, UM2 harboring pYZ55 ($katG^+$); lane 6, UM2 harboring pYZ56 (lacZ'::katG).

DETAILED DESCRIPTION OF PREFERRED EMBODIMENT

The recent emergence of large numbers of strains of M. tuberculosis showing multidrug resistance in the United States is a most alarming development given the extreme contagiousness of this organism. This danger has been strikingly illustrated by several small tuberculosis epidemics in which a single patient infected with MDR M. tuberculosis has infected both HIV-positive individuals, prison guards and healthy nursing staff (CDC 1990, 1991;

Daley et al., 1992; Snider and Roper, 1992). Given the gravity of the current worldwide HIV epidemic, it is conceivable that if AIDS patients in the West, like those in Africa, were to be infected with MDR M. tuberculosis strains (rather than members of the M. avium/M. intracellulare complex) widespread dissemination of the disease would result.

Isoniazid (INH) is a bactericidal drug which is particularly potent against the tuberculosis group of mycobacteria - Mycobacterium tuberculosis, M. bovis, and M. africanum - and, in consequence, it has been particularly effective in the treatment of tuberculosis. Standard anti-tuberculosis regimens generally include INH and rifampicin, often in combination with the weaker drugs, pyrazinamide, ethambutol or streptomycin. Besides its use in therapy INH is also given to close contacts of patients as a prophylactic measure.

INH-resistant mutants of M. tuberculosis, the agent of the human disease, show two levels of resistance: low (1 to 10 μg/ml) and high (10 to 100 μg/ml). INH-resistance is often associated with loss of catalase activity and virulence. Recently, owing to the AIDS epidemic, increased homelessness and declining social conditions, tuberculosis has reemerged as a major public health problem in developed countries, particularly the USA. An alarming feature of the disease today is the emergence of multiple drug-resistant organisms and rapid nosocomial transmission to health care workers and HIV-infected patients. This has prompted CDC to propose new recommendations for the treatment of multiple resistant strains (at least INH and rifampicin) and the prevention of transmission. To obtain fresh insight into the

problem of INH-resistance and to develop a rapid diagnostic test the following study was performed.

Clearly, it is essential to understand the mechanisms of resistance to INH and rifampicin, the main anti-tuberculosis agents, as this will allow novel chemotherapeutic strategies to be developed and facilitate the design of new compounds active against MDR strains.

This invention demonstrates that it is the catalase-peroxidase enzyme, HPI, which is the INH target, and it is suggested that this enzyme alone mediates toxicity. Compelling evidence of this conclusion was obtained by expression of the M. tuberculosis katG gene in a catalase-negative mutant of E. coli as this resulted in this bacterium becoming sensitive to INH. Moreover, the isolation of the M. tuberculosis INH-sensitivity gene, katG, is important as it will facilitate the rapid detection of INH-resistant strains by means of hybridization and PCR-based approaches. The high frequency of katG deletions in clinical strains, as shown here, should simplify this procedure.

Identification of an M. tuberculosis gene involved in INH-sensitivity

A heterologous approach was employed to isolate M. tuberculosis gene(s) involved in INH-sensitivity. BH1 is a spontaneous mutant of the easily transformable M. smegmatis strain MC^2155 (Snapper et al., 1990), that is resistant to 512 $\mu g/ml$ of the INH and lacks catalase-peroxidase activity (Heym et al., 1992). As there is a strict correlation between INH-sensitivity and these enzyme activities, transformation of BH1 with a

plasmid carrying the appropriate gene from M. tuberculosis should lead to their restoration and concomitant INH-sensitivity.

Consequently, DNA was prepared from a pool of M. tuberculosis shuttle cosmids in Escherichia coli and introduced into BH1 by electro-transformation. Over 1000 kanamycin-resistant transformants were then scored for INH-sensitivity, and four clones that failed to grow on medium containing 32 g/ml of INH, the MIC from wild type strain MC^2155, were obtained.

After re-transformation of BH1, only one of these, pBH4, consistently conferred the INH-sensitive phenotype. Restriction digests with BamHI, KpnI, NotI, ClaI and HindIII showed the M. tuberculosis chromosomal DNA carried by pBH4 to be about 30 kb in size. A map produced with the last three enzymes is presented in Fig. 1.

When pBH4 was used as a hybridization probe to detect homologous clones in the library, a further eight shuttle cosmids were isolated. On transformation into BH1, five of these (T35, T646, T673, T79, T556) restored INH-sensitivity, and showed similar restriction profiles to pBH4. In particular, a KpnI fragment of 4.5 kb was present in all cases.

Attempts to subclone individual BamHI fragments did not give rise to transformants capable of complementing the lesion in BH1 suggesting that a BamHI site might be located in the gene of interest. In contrast, pBH5, a derivative of pBH4, was constructed by deletion of EcoRI fragments and this showed that a 7 kb segment was not required for restoration of INH-sensitivity.

Transformants harboring shuttle cosmids that

complemented the INH-resistant mutation of BH1 were examined carefully and the MICs for several antibiotics were established. In all cases, the MIC for INH had been reduced from 512 to 8 μg/ml, a value lower than that of the sensitive strain MC^2155 (32 μg/ml). This hypersensitive phenotype suggested that the recombinant clones might be overproducing an enzyme capable of enhancing INH-toxicity. Enzymological studies showed that these transformants all produced about 2-fold more peroxidase and catalase than the wild type strain MC^2155, which is INH-sensitive.

In addition to INH, many MDR-strains of M. tuberculosis are no longer sensitive to rifampicin, streptomycin, ethambutol and pyrazinamide. To examine the possibility that there might be a relationship between resistance to INH and these compounds, the MICs of several drugs for various M. smegmatis strains and their pBH4 transformants were determined, but no differences were found.

Cloning the M. tuberculosis catalase gene

A 45-mer oligonucleotide probe was designed based on the primary sequences of highly conserved regions in the catalase-peroxidase enzymes, HPI, of E. coli (Triggs-Raine et al., 1989), and Bacillus stearothermophilus (Loprasert et al., 1988). When genomic blots of M. tuberculosis DNA were probed with this oligonucleotide, specific bands were detected in most cases. As KpnI generated a unique fragment of 4.5 kb that hybridized strongly, this enzyme was used to produce a size selected library in pUC19.

Upon screening with the oligonucleotide probe, an appropriate clone, pYZ55, was obtained. A restriction

map of the insert DNA is presented in Fig. 1 where it can be seen that this corresponds exactly to part of pBH4. Independent confirmation was also obtained by cross-hybridization.

By means of various subcloning experiments the smallest fragment expressing M. tuberculosis catalase-peroxidase activity in E. coli was found to be a 2.5 kb EcoRV-KpnI fragment which, as expected, contained a cleavage site for BamHI. Partial DNA sequence analysis showed that the katG gene carried by pYZ56 encodes a catalase-peroxidase enzyme that is highly homologous to the HPI enzymes of E. coli and B. stearothermophilus:

M.tuberculosis APLNSWPDNASLDKARRLLWPSKKKYGKKLSWADLIV
E.coli *********V************I*Q***Q*I*****FI
B.stearothermophilus**********N******C*GR**RNT*T*-*LGPICS

(Fig. 2; Triggs-Raine et al., 1988); (Loprasert et al., 1988). Identical residues are indicated by *. HPI activity was detected in both E. coli and M. smegmatis by staining (see below).

Catalase-peroxidase involvement in INH-sensitivity

Having cloned the M. tuberculosis katG gene, it was of immediate interest to investigate the genetic basis of the association between catalase-negativity and isoniazid resistance. A series of constructs was established in the shuttle vector pBAK14 and used to transform the INH-resistant M. smegmatis mutant BH1. Only those plasmids carrying a complete katG gene produced HPI and restored INH-sensitivity. The smallest of these, pBAK14, carried a 2.5 kb EcoRV-KpnI fragment thus demonstrating that the 2 kb region upstream of katG was not involved, and that catalase-peroxidase activity alone was

sufficient to render mycobacteria susceptible to INH.

Cell-free extracts were separated by non-denaturating polyacrylamide gel electrophoresis and stained for peroxidase and catalase activity. Under these conditions, the M. tuberculosis enzyme gave two bands of peroxidase activity (lane 1) which comigrated with catalase activity (Heym et al., 1992).

When introduced into E. coli, the katG gene directed the synthesis of the same proteins, whereas pYZ56 produced proteins slightly larger in size. This is due to the construction of an in-frame lacZ::katG gene fusion. Activity stains were also performed with cell extracts of M. smegmatis. The presence of the katG gene from the M. tuberculosis in BH1 led to the production of catalase-peroxidase enzyme, which displayed the same electrophoretic mobility as the enzyme made in M. tuberculosis, or in E. coli, and the native HPI of M. smegmatis.

Basis of INH-resistance in M. tuberculosis

It has been known for many years that a subset of INH--resistant strains, particularly those resistant to the highest drug concentrations, are of lower virulence in the guinea pig and devoid of catalase activity. Genomic DNA was prepared from several clinical isolates of M. tuberculosis and analyzed by Southern blotting using the 4.5 kb KpnI fragment as a probe. In two highly resistant strains, B1453 and 24, the catalase gene has been deleted from the chromosome whereas in others (Fig. 3), such as strain 12, showing low level resistance it is still present but not expressed. Additional studies showed that the region immediately prior to katG was highly

prone to rearrangements.

M. tuberculosis HPI renders E. coli sensitive to INH

To determine whether the HPI enzyme of M. tuberculosis could confer INH sensitivity on E. coli, a series of catalase mutants was transformed with pYZ56 and the MICs determined. Wild type strains were not susceptible to INH, but mutants lacking both endogenous catalase activities, but harboring pYZ56, showed growth inhibition when high levels of INH (500 μg/ml) were present, whereas untransformed strains were insensitive.

For purposes of this invention, a plasmid containing the restriction endonuclease map shown in Fig. 1 was deposited in strain with the National Collection of Cultures of Microorganisms (C.N.C.M.) of the Institut Pasteur, in Paris, France on May 18, 1992, under culture collection accession No. I-1209. This plasmid contains the nucleic acid sequence of the invention, namely, the 4.5 kb KpnI-KpnI fragment of plasmid pYZ56 having the BamHI cleavage site in ~~the~~ fragment.

In general, the invention features a method of detecting the presence of isoniazid-resistant Mycobacterium tuberculosis in a sample including providing at least one DNA or RNA probe capable of selectively hybridizing to isoniazid-sensitive Mycobacterium tuberculosis DNA to form detectable complexes. Detection is carried out with a sample under conditions which allow the probe to hybridize to isoniazid-sensitive Mycobacterium tuberculosis DNA present in the sample to form hybrid complexes and detecting the hybrid complexes as an indication of the presence of isoniazid-sensitive Mycobacterium

tuberculosis in the sample. (The term "selectively hybridizing", as used herein, refers to a DNA or RNA probe which hybridizes only to isoniazid-sensitive Mycobacterium tuberculosis and not to isoniazid-insensitive Mycobacterium tuberculosis.) The sample can be comprised of the Mycobacterium tuberculosis cells or a portion of the cells or cell contents enriched in Mycobacterium tuberculosis nucleic acids, especially DNA. Hybridization can be carried out using conventional hybridization reagents. The particular hybridization conditions have not been found to be critical to the invention.

More particularly, DNA sequences from Mycobacterium tuberculosis can be analyzed by Southern blotting and hybridization. The techniques used for the present invention are described in Maniatis et al. (1989). DNA fragments can be separated on agarose gels and denatured in situ. The fragments can then be transferred from the gel to a water insoluble solid, porous support, such as a nitrocellulose filter, a nylon membrane, or an activated cellulose paper, where they are immobilized for example, the Hybond® membrane commercialized by Amersham can be used. After prehybridization to reduce non-specific hybridization with the probe, the solid support is hybridized to the nucleic acid probe of the invention. The solid support is washed to remove unbound and weakly binding probe, and the resulting hybrid duplex molecule is examined. A convenient alternative approach is to hybridize oligonucleotides to the DNA denatured in the gel.

The amount of labeled probe which is present in the hybridization solution will vary widely, depending upon

the nature of the label, the amount of the labeled probe which can reasonably bind to the filter, and the stringency of the hybridization. Generally, substantial excesses of the probe over stoichiometric will be employed to enhance the rate of binding of the probe to the fixed DNA.

Various degrees of stringency of hybridization can be employed. The more severe the conditions, the greater the complementarity that is required for hybridization between the probe and the polynucleotide for duplex formation. Severity can be controlled by temperature, probe concentration, probe length, ionic strength, time, and the like. Conveniently, the stringency of hybridization is varied by changing the polarity of the reactant solution. Temperatures to be employed can be empirically determined or determined from well known formulas developed for this purpose.

Unlike Southern hybridization where DNA fragments are transferred from an agarose gel to a solid support, the method of the invention can also be carried out by oligonucleotide hybridization in dried agarose gels. In this procedure, the agarose gel is dried and hybridization is carried out in situ using an oligonucleotide probe of the invention. This procedure is preferred where speed of detection and sensitivity may be desirable. The procedure can be carried out on agarose gels containing genomic or cloned DNA of Mycobacterium tuberculosis.

In addition, the method of this invention can be carried out by transfer of Mycobacterium tuberculosis DNA from polyacrylamide gels to nylon filters by electroblotting. Electroblotting may be desirable where

time is of the essence, because electroblotting is typically faster than capillary blotting developed to transfer DNA from agarose gels. This method can be carried out in conjunction with UV-crosslinking. The polyacrylamide gel containing the samples to be tested is placed in contact with an appropriately prepared nylon filter. These are then sandwiched into an electroblotting apparatus and the DNA is transferred from the gel onto the filter using electric current. After a buffer rinse, the filter is ready to be prehybridized and hybridized or UV-crosslinked.

The method of the invention can be carried out using the nucleic acid probe of the invention for detecting Mycobacterium tuberculosis resistant to isoniazid. The probe can be detected using conventional techniques.

The method of the invention can also detect point mutations in the KatG gene, as well as a partial deletion of that gene.

The nucleotides of the invention can be used as probes for the detection of a nucleotide sequence in a biological sample of M. tuberculosis. The polynucleotide probe can be labeled with an atom or inorganic radical, most commonly using a radionuclide, but also perhaps with a heavy metal. Radioactive labels include ^{32}P, ^{3}H, ^{14}C, or the like. Any radioactive label can be employed, which provides for an adequate signal and has sufficient half-life. Other labels include ligands that can serve as a specific binding member to a labeled antibody, fluorescers, chemiluminescers, enzymes, antibodies which can serve as a specific binding pair member for a labeled ligand, and the like. The choice of the label will be governed by the effect of the label on the rate of

hybridization and binding of the probe to the DNA or RNA. It will be necessary that the label provide sufficient sensitivity to detect the amount of DNA or RNA available for hybridization.

In preferred embodiments of the invention, the probe is labeled with a radioactive isotope, e.g., ^{32}P or ^{125}I, which can be incorporated into the probe, e.g., by nick-translation.

In other preferred embodiments, the probe is labeled with biotin, which reacts with avidin to which is bonded a chemical entity which, when the avidin is bonded to the biotin, renders the hybrid DNA complex capable of being detected, e.g., a fluorophore, which renders the hybrid DNA complex detectable fluorometrically; an electron-dense compound capable of rendering the hybrid DNA complexes detectable by an electron microscope; an antibody capable of rendering the hybrid DNA complexes immunologically detectable; or one of a catalyst/substrate pair capable of rendering the hybrid DNA complexes enzymatically detectable. Prior to contacting the bacteria with the probe, the M. tuberculosis bacteria can be lysed to release their DNA, which is then denatured and immobilized on an appropriate solid, DNA-binding support, such as a nitrocellulose membrane.

Another detection method, which does not require the labeling of the probe, is the so-called sandwich hybridization technique. In this assay, an unlabeled probe, contained in a single-stranded vector, hybridizes to isoniazid-sensitive Mycobacterium tuberculosis DNA, and a labeled, single-stranded vector, not containing the probe, hybridizes to the probe-containing vector, labeling the whole hybrid complex.

The sequences of the invention were derived by dideoxy-nucleotide sequencing. The base sequences of the nucleotides are written in the 5'-----> 3' direction. Each of the letters shown is a conventional designation for the following nucleotides:

A	Adenine
G	Guanine
T	Thymine
C	Cytosine.

The nucleotides of the invention can be prepared by the formation of 3'-----> 5' phosphate linkages between nucleoside units using conventional chemical synthesis techniques. For example, the well-known phosphodiester, phosphotriester, and phosphite triester techniques, as well as known modifications of these approaches, can be employed. Deoxyribonucleotides can be prepared with automatic synthesis machines, such as those based on the phosphoramidite approach. Oligo- and polyribonucleotides can also be obtained with the aid of RNA ligase using conventional techniques.

The nucleotides of the invention are in a purified form. For instance, the nucleotides are free of human blood-derived proteins, human serum proteins, viral proteins, nucleotide sequences encoding these proteins, human tissue, and human tissue components. In addition, it is preferred that the nucleotides are free of other nucleic acids, extraneous proteins and lipids, and adventitious microorganisms, such as bacteria and viruses.

This invention of course includes variants of the nucleotide sequences of the invention or serotypic variants of the probes of the invention exhibiting the same selective hybridization properties as the probes

identical herein.

The nucleotide sequences of the present invention can be employed in a DNA amplification process known as the polymerase chain reaction (PCR). See, e.g., Kwok et al. (1987). PCR is advantageous because this technique is rapid.

DNA primer pairs of known sequence positioned 10-300 base pairs apart that are complementary to the plus and minus strands of the DNA to be amplified can be prepared by well known techniques for the synthesis of oligonucleotides. One end of each primer can be extended and modified to create restriction endonuclease sites when the primer is annealed to the PBMC DNA. The PCR reaction mixture can contain the PBMC DNA, the DNA primer pairs, four deoxyribonucleoside triphosphates, $MgCl_2$, DNA polymerase, and conventional buffers. The DNA can be amplified for a number of cycles. It is generally possible to increase the sensitivity of detection by using a multiplicity of cycles, each cycle consisting of a short period of denaturation of the PBMC DNA at an elevated temperature, cooling of the reaction mixture, and polymerization with the DNA polymerase.

Amplified sequences can be detected by the use of a technique termed oligomer restriction (OR). Single-strand conformation polymorphism (SSCP) analysis can be used to detect DNA polymorphisms and point mutations in a variety of positions in DNA fragments. See, Saiki et al. (1985); Orita et al. (1989). For example, after amplification, a portion of the PCR reaction mixture can be separated and subjected to hybridization with an end-labeled nucleotide probe, such as a ^{32}p labelled adenosine triphosphate end-labeled

probe. In OR, an end-labeled oligonucleotide probe hybridizes in solution to a region of the amplified sequence and, in the process, reconstitutes a specific endonuclease site. Thus, hybridization of the labeled probe with the amplified katG sequence yields a double-stranded DNA form that is sensitive to selective restriction enzyme digestion. After restriction with an endonuclease, the resulting samples can be analyzed on a polyacrylamide gel, and autoradiograms of the portion of the gel with the diagnostic labeled fragment can be obtained. The appearance of a diagnostic fragment (e.g., 10-15 bases in length) in the autoradiogram indicates the presence of katG sequences in the PBMCs.

Since it may be possible to increase the sensitivity of detection by using RNA instead of chromosomal DNA as the original template, this invention contemplates using RNA sequences that are complementary to the DNA sequences described herein. The RNA can be converted to complementary DNA with reverse transcriptase and then subjected to DNA amplification.

EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids

Table 1 outlines the properties of the bacterial strains and plasmids used in this invention.

Table 1. Bacterial Strains And Plasmids

Strains/plasmids	Characteristics
E. coli NM554	
E. coli TG1	supE hsd5 thi delta (lac-proAB) [traD36 proAB+ lacI^{q} lacZ delta M15]
E. coli UM2	KatE
E. coli UM255	KatE
M. tuberculosis H37Rv	Virulent strain originally isolated from tuberculosis patient
M. tuberculosis 12	Clinical isolate resistant to low levels of INH (1-2 μg/ml)
M. tuberculosis B1453	Clinical isolate resistant to high levels of INH (>50 μg/ml)
M. tuberculosis 24	Clinical isolate resistant to high levels of INH (>50 μg/ml)
M. tuberculosis 79112	Clinical isolate sensitive to INH
M. tuberculosis 12646	Clinical isolate sensitive to INH
M. tuberculosis 79665	Clinical isolate sensitive to INH
M. smegmatis MC^{2}155	MC^{2}6 het
M. smegmatis BH1	MC^{2}155 het katG

Plasmids

pBH4	Shuttle cosmid, katG+, based on pYUB18
pBH5	Deleted version of pBH4, katG+, (7 kb-EcoRI)
pYZ55	pUC19 derivative with 4.5 kb KpnI fragment, kat+
pYZ56	pUC19 derivative with 2.5 kb EcoRV-KpnI fragment (kat+)
pYZ57	pUC19 derivative with 3.1 kb KpnI-BamHI fragment, kat-
pBAK14	Mycobacterial shuttle vector (Zhang et al., 1991)
pBAK15	Mycobacterial shuttle vector carrying 4.5 kb KpnI fragment (kat+)
pBAK16	Mycobacterial shuttle vector carrying 2.5 kb EcoRV-KpnI fragment (kat^+)
pBAK17	Mycobacterial shuttle vector carrying 3.1 kb KpnI-BamHI fragment (kat-)

The *M. tuberculosis* H37 RV genomic library was constructed in the shuttle cosmid pYUB18 (Snapper et al., 1988) and kindly supplied by Dr. W. R. Jacobs. Other shuttle vectors employed were pYUB12 (Snapper et al., 1988) and pBAK14 (Zhang et al., 1991).

Microbiological techniques and enzymology

Details of antibiotics used, growth conditions, enzymology and MIC determinations can be found in Heym et al., (1992).

Nucleic acid techniques

Standard protocols were used for subcloning, Southern blotting, DNA sequencing, oligonucleotide biosynthesis, etc. (Maniatis et al., 1989; Eiglmeier et al., 1991).

Activity staining

The preparation of cell-free extracts of E. coli and mycobacteria has been described (Heym et al., 1992; Zhang et al., 1991). Native protein samples were separated by polyacrylamide gel electrophoresis as described by Laemmli (1970) except that SDS was omitted from all buffers, samples were not boiled and betamercaptoethanol was not included in the sample buffer. After electrophoresis of 50 - 100 μg protein samples on 7.5% polyacrylamide gels, catalase activity was detected by soaking the gel in 3mM H_2O_2 for 20 minutes with gentle shaking. An equal volume of 2% ferric chloride and 2% potassium ferricyanide was added and clear bands of catalase activity revealed by illumination with light. Peroxidase activity was detected as brown bands after soaking gels in a solution containing 0.2 - 0.5 mg/ml diaminobenzidine and 1.5 mM H_2O_2 for 30 - 120 minutes.

To generate a highly toxic compound it seems most likely that the M. tuberculosis HPI enzyme peroxidatively activates INH (Youatt, 1969; Gayathri-Devi et al., 1975). Now that the katG gene has been isolated and characterized, it should be possible to make new derivatives of INH, which can be activated in a similar manner.

Example 1

Point mutations in the katG gene associated with the isoniazid-resistance of M. tuberculosis

It has been shown in a recent study that the catalase-peroxidase of Mycobacterium tuberculosis, encoded by the katG gene, is involved in mediating the toxicity of the potent anti-tuberculosis drug isoniazid or INH. Mutants resistant to clinical levels of INH show reduced catalase-peroxidase activity and, in some cases, this results from the deletion of the katG gene from the chromosome.

Transformation of INH-resistant strains of Mycobacterium smegmatis and M. tuberculosis with the cloned katG gene leads to restoration of drug-sensitivity. Expression of katG in some strains of Escherichia coli renders this naturally resistant organism susceptible to high concentrations of INH.

As some INH-resistant clinical isolates of M. tuberculosis have retained an intact katG gene, the molecular basis of their resistance was investigated. This study was facilitated by the availability of the nucleotide sequence of a 4.7 kb KpnI fragment from the katG region of the chromosome as this allowed primers suitable for PCR analysis to be designed. Eleven pairs of oligonucleotide primers were synthesized (see Table 2) and used to generate PCR-products, of around 280 bp, that covered the complete katG gene and some of the flanking sequences. In control experiments all experiments all eleven primer pairs generated PCR products of the expected size, highly suitable for SSCP-analysis, so a panel of 36 INH-resistant strains of M. tuberculosis, of Dutch or French origin, was examined. Many of these strains are multidrug resistant and were isolated from patients who were HIV-seropositive.

Table 2. Sequences of primer pairs used for PCR-SSCP analysis of the *katG* gene of *M. tuberculosis*

		5'	3'	Length	G+C(%)	Tm	Production
Primer Pair #	1						288
OLIGO1:	GCGGGGTTATCGCOGATG	1765	1782	18	66	61.8	
OLIGO2:	GCCCTCGACGGGGTATTTC	2052	2034	19	63	61.9	
Primer Pair #	2						300
OLIGO1:	AACGGCTGTCCCGTOGTG	2008	2025	18	66	61.9	
OLIGO2:	GTCGTGGATGCGGTAGGTG	2307	2289	19	63	61.9	
Primer Pair #	3						280
OLIGO1:	TCGACTTGACGCCCTGACG	2169	2187	19	63	61.9	
OLIGO2:	CAGGTCOGOOCATGACAG	2448	2431	18	66	61.9	
Primer Pair #	4						284
OLIGO1:	OCACAACGCCAGCTTCGAC	2364	2382	19	53	61.9	
OLIGO2:	GGTTCACGTAGATCAGCCCC	2647	2628	20	50	61.9	
Primer Pair #	5						288
OLIGO1:	GCAGATGGGGCTGATCTACG	2622	2641	20	60	51.9	
OLIGO2:	ACCTCGATGOOGCTGGTG	2909	2892	18	66	51.9	
Primer Pair #	6						286
OLIGO1:	GCTGGAGCAGATGGGCTTG	2829	2847	19	63	61.9	
OLIGO2:	ATCCACCOGCAGCGAGAG	3114	3097	18	66	61.9	
Primer Pair #	7						297
OLIGO1:	GCCACTGACCTCTCGCTG	3088	3105	18	66	61.9	
OLIGO2:	CGCCCATGCGGTCGAAAC	3384	3367	18	66	61.9	

Primer Pair #	8						
OLIGO1:	GCGAAGCAGATTGCCAGCC	3304	3322	19	63	61.9	285
OLIGO2:	ACAGCCACCGAGCACGAC	3588	3571	18	66	61.9	
Primer Pair #	9						
OLIGO1:	CAAACTGTCCTTCGCCGAOC	3549	3568	20	60	61.9	281
OLIGO2:	CACCTACCAGCACCGTCATC	3829	3810	20	60	61.9	
Primer Pair #	10						
OLIGO1:	TGCTCGACAACGCGAACCTG	3770	3789	20	60	61.9	280
OLIGO2:	TCCGAGTTGGACCOGAAGAC	4049	4030	20	60	61.9	
Primer Pair #	11						
OLIGO1:	TACCAGGGCAAGGATGGCAG	3973	3992	20	60	61.9	280
OLIGO2:	GCAAACACCAGCACCCCG	4252	4235	18	66	61.9	

{#courier10}

Two of them gave no PCR fragment, with any of the primers used, indicating that katG had been deleted. The remaining 34 strains all yielded the expected PCR products and these were analyzed on SSCP gels so that possible point mutations could be detected. In 20 cases, abnormal strand mobility was observed, compared to that of katG from drug-sensitive M. tuberculosis, suggesting that mutational events had indeed occurred. The approximate locations of the mutations, as delimited by the PCR primers, are shown in Table 3.

Table 3. Preliminary results of PCT-SSCP analysis of *katG* from *M. tuberculosis* strains
x denotes altered mobility; del denotes deletion

N°	Strain	MIC (INH)	1 1765-2034	2 2008-2289	3 2169-2431	4 2364-2628	5 2622-2892	6 2829-3097	7 3088 3367
1/37	9488	1							X
2	9577	1							
3	9112	10							
4	9247	1							
5	9200	1					X		
6	9116	1							
7/31	9106	1					X		X
8	9291	1							X
9/10	9412	1	X						
11/12	9435	1							
13	9428	1			X				
14	9441	1			X				X
15/16	9444	1			X				X
17/18	9445	1							
19/20	9330	0,2			X				
21/22	9420	0,2							
23	9262	0,2							
24/38	9523	1					X		
25	9592	10			X				
26	9553	10							
27	9485	10					X		
28	9181	1			X		X		
29	9363	1							X
30	9465	1			X				
32	9178	0,2							
33	9468	0,2							
34	9218	0,2							

N°	Strain	MIC (INH)	1 1765-2034	2 2008-2289	3 2169-2431	4 2364-2628	5 2622-2892	6 2829-3097	7 3088 3367
33	9468	0,2							
34	9218	0,2							
35	9503	0,2					X		
39	9582	1							
41	H37Rv	-							
42	Ass	-							
43	Mou	-	del	del	del	del	del	del	del
44	13632	>20	del	del	del	del	del	del	del
45	13549	>5							
46	13749	>20							X
47	14006	10							
48	13711	>5					X		
49	13681	>5							
50	14252	>5							

On examination of a 200 bp segment of the katG gene from five independent strains (9188, 9106, 9441, 9444, 9363), a single base difference was found. This was the same in all cases, a G to T transversion at position 3360, resulting in the substitution of Arg-461 by Leu. Thus, in addition to inactivation of katG, INH-resistance can stem from mis-sense mutations that result in an altered catalase peroxidase. This mutation may define a site of interaction between the drug and the enzyme. The results of DNA sequence studies with the remaining mutants are eagerly awaited.

Another conclusion that can be drawn from this study concerns the molecular basis of the multidrug resistance associated with various M. tuberculosis strains. The same mutations are found irrespective of whether a given patient is seropositive or seronegative for HIV. For example, strain 9291, isolated from an HIV-seropositive tuberculosis patient, harbors mutations conferring resistance to INH, rifampin and streptomycin in the katG (R461L), rpoB (S425L) and rpsL (K42R) genes, respectively. The same mutations have been found separately, or in combination, in strains from HIV-seronegative individuals. This means that, for the set of strains studied, there is no novel, single mechanism conferring resistance to several drugs, but rather,

multidrug resistance results from the accumulation of mutations in the genes for distinct drug targets.

Example 2

Nucleotide sequence and chromosomal location of the katG locus of M. tuberculosis

Bacterial strains, plasmids and growth conditions. The following bacterial strains from our laboratory collections were used in this study: M. tuberculosis H37Rv; M. smegmatis MC^2155 (Snapper et al., 1990); E. coli K-12 UM2 (katE katG; Mulvey et al., 1988). The recombinant plasmids, pYZ55 (pUC19, $katG^+$), pYZ56 (pUC19, lacZ'::katG) and the shuttle clones, pBH4 (pYUB18, $katG^+$) and pBAK-KK- (pBAK14, $katG^+$) have been described recently (Zhang et al. 1992, Nature) and the katG locus of M. tuberculosis is schematized in Fig. 5. Mycobacteria were grown at 37°C in Middlebrook 7H9 medium, while E. coli strains were cultivated in L-broth, with appropriate enrichments and antibiotics.

Nucleic acid techniques. Standard techniques were employed for the preparation, labelling and hybridization of DNA (Eiglmeier et al. 1991; Zhang et al. 1992, Infect. Immun.; Zhang et al. 1992, Nature). A shotgun library of random fragments of pYZ55 was prepared in M13mp18 as described previously (Garnier et al., 1986) and sequenced using the modified dideoxy technique (Biggin et al. 1983). Sequences were compiled and assembled into contigs using SAP, and analyzed with NIP, SIP and PIP (Staden 1987) running on a Vax 3100 workstation. Gap closure was obtained by using synthetic oligonucleotide primers, synthesized on an ABI 381 apparatus, and T7 DNA polymerase (Pharmacia) to obtain sequences directly from pYZ55. To search for related sequences in the GenBank database (release 73.1) the FASTA (Pearson et al. 1988) and BLAST (Altschul et al. 1990)

programs were used. The PROSITE (Bairoch 1992) catalog was screened to detect possible motifs present in protein sequences and alignments were done with the PILEUP and PRETTY modules of the GCG sequence analysis package (Devereux et al. 1984).

Western blotting and catalase-peroxidase activity staining. Immunoblotting of polypeptides resolved by SDS-polyacrylamide gel electrophoresis and detection with polyclonal antibodies (purchased from DAKO) raised against M. bovis BCG, were as described (Zhang et al. 1992, Infect. Immun., Nature, Mol. Microbiol.). Procedures for detecting catalase and peroxidase activities have been outlined recently (Heym et al. 1992; Zhang et al. 1992, Nature).

RESULTS

Nucleotide sequence of the katG locus of M. tuberculosis. In previous studies, the complete katG gene was cloned independently in E. coli on a shuttle cosmid, pBH4, and on a 4.5 kb KpnI restriction fragment thus giving rise to pYZ55 (Fig. 5; Zhang et al. 1992, Nature). The structural gene for catalase-peroxidase was subsequently localized to a 2.5 kb EcoRV - KpnI fragment by sub-cloning. To deduce the primary structure of this important enzyme and thereby gain some insight into its putative role in the conversion of INH into a potent anti-tuberculous derivative, the nucleotide sequence of the complete insert from pYZ55 was determined. This was achieved by the modified dideoxy-shotgun cloning procedure (Biggin et al. 1993) and gaps between the contigs were closed by using specific primers.

On inspection of the resultant sequence which is shown in Fig. 6A, the 4.5 kb fragment was found to contain 4795 nucleotides with an overall dG+dC content of 64.4%. When this was analyzed for the presence of open reading frames,

with high coding-probability values, a single candidate was detected and, from its size, composition and location, this was identified as katG. The absence of any additional open reading frames, on either strand of the KpnI fragment, ruled out the possibility that genes other than katG were involved in conferring INH-susceptibility.

Further analysis of the sequence showed katG to be preceded by two copies of a 700 bp direct repeat which were 68% identical, with the longest stretch of identity comprising 58 bp (Fig. 6B). When the databases were screened with this sequence no significant homologies were detected. To test the possibility that it could correspond to a new repetitive element in M. tuberculosis, a 336 bp probe, encompassing the 58 bp repeat, was used to probe a partially-ordered cosmid library. Positive hybridization signals were only obtained from clones that were known to carry katG. Likewise, a single restriction fragment was detected in Southern blots of M. tuberculosis DNA digested with restriction enzymes BamHI, KpnI and RsrII thereby indicating that this repetitive sequence is not dispersed.

Chromosomal location of katG. As part of the M. tuberculosis genome project, most of the genes for which probes are available have been positioned on the contig map. From the series of overlapping cosmids shown in Fig. 5 it can be seen that the markers linked to katG are LL105 and fbpB encoding an anonymous antigen and the putative fibronectin binding protein, or alpha antigen (Matsuo et al. 1988), respectively. None of the known insertion sequences IS6110 and IS1081 (Collins et al. 1991; McAdam et al. 1990; Thierry et al. 1990, J. Clin. Microbiol.; Thierry et al. 1990, Nucleic Acids Res.), map to this area of the chromosome although the region upstream of katG is densely populated

with copies of the major polymorphic tandem repeat, MPTR (Hermans et al. 1992; Zhang and Young 1993).

Presence of katG homologues in other mycobacteria. INH is exquisitely potent against members of the tuberculosis complex yet shows little, if any, activity against other mycobacteria. To determine whether genes homologous to katG were present in other mycobacteria Southern blots of DNA digested with RsrII were hybridized with a probe prepared from a 2.5 kb EcoRV-KpnI restriction fragment carrying katG from M. tuberculosis. Under conditions of high stringency good signals were obtained from M. leprae and M. avium (Fig. 7) while barely discernible hybridization was observed with M. gordonae and M. szulgai. It has been shown recently that katG homologues are also present in M. Smegmatis and M. aurum (Heym et al. 1992).

Predicted properties of catalase-peroxidase from M. tuberculosis. The primary structure of catalase-peroxidase, deduced from the nucleotide sequence of katG, is shown in Fig. 6. The enzyme is predicted to contain 735 amino acids, and to display a molecular weight of 80,029 daltons. A protein of this size has been observed in M. tuberculosis, and both recombinant M. smegmatis and E. coli (see below).

Primary structures are available for several other bacterial catalase-peroxidases including those from E. coli, salmonella typhimurium and Bacillus stearothermophilus (Loewen et al. 1990; Loprasert et al. 1988; Triggs-Raine et al. 1988) and these have been shown to be distantly related to yeast cytochrome c peroxidase (Welinder 1991). As the crystal structure of the latter has been determined (Finzel et al. 1984) this can be used to interpret the sequences of the bacterial enzymes. The M. tuberculosis enzyme shows 53.3% conservation with the enterobacterial HPI enzymes, and

shares 45.7% identity with the protein from B. stearothermophilus. An alignment of the sequences of these four enzymes is shown in Fig. 8, along with that of yeast cytochrome c peroxidase (Welinder 1991). It is apparent that the NH_2 terminus, which has no counterpart in the yeast enzyme, is the most divergent part suggesting that this domain of the protein can tolerate extensive deviation and is not required for catalysis. Experimental support for this interpretation is provided in the form of a LacZ-KatG fusion protein which contains an additional 40 amino acid residues (Fig. 9, lane 6; Zhang et al. 1992, Nature). Addition of this NH_2-terminal segment does not noticeably interfere with either the catalase or peroxidase reactions effected by KatG as judged by activity staining (Zhang et al. 1992, Nature).

Bacterial catalase-peroxidases are believed to have evolved by means of a gene duplication event and consist of two modules, both showing homology to the yeast enzyme, fused to a unique NH_2-terminal sequence of about 50 amino acid residues (Welinder 1991). The M. tuberculosis enzyme conforms to this pattern and when searched for internal homology using SIP (Staden 1987) it was clear that the region between residues 55-422 was related to the carboxy terminal domain, consisting of amino acids 423-735. Only one of the two active site motifs typical of peroxidases, present in the PROSITE catalog (Bairoch 1992) was found when the M. tuberculosis catalase-peroxidase primary structure was screened as there are two deviations from the consensus around His^{269} where the second motif should be. (Consensus pattern for peroxidase 1: [DET]-[LIVMT]-x(2)-[LIVM]-[LIVMSTAG]-[SAG]-[LIVMSTAG]-H-[STA]-[LIVMFY]; consensus pattern for peroxidase 2: [SGAT]-x(3)-[LIVMA]-R-[LIVMA]-x-[FW]-H-x-[SAC]; (Bairoch

1992). In addition, a possible ATP-binding motif (G-x-x-x-x-G-K-T) was detected (Balroch 1992) but as this partially overlaps the active site its presence may be purely fortuitous (Fig. 8).

By analogy with yeast cytochrome c peroxidase (Welinder 1991), it was possible to predict a number of structurally and catalytically important residues all of which are located in the NH_2-terminal repeat. His^{269} should serve as the fifth ligand of the heme-iron while Asp^{380} should be its hydrogen-bonded partner. Other residues predicted to be involved in active site modulation and H_2O_2 binding are Arg^{104}, Trp^{107}, His^{108}, Asn^{138}, Thr^{274} and His^{275} (Fig. 4). According to Welinder's predictions (Welinder 1991), Trp^{320} should be a key residue and be required for forming the protein-radical site (Sivaraja et al. 1989).

Antibody response to M. tuberculosis KatG. To evaluate the possible value of KatG as an immunogen, Western blots were probed with anti-serum raised against M. bovis BCG in rabbits. As shown in Fig. 9, the 80 kD catalase-peroxidase is one of the prominent antigens recognized in cell-free extracts of M. tuberculosis, and M. smegmatis expressing the cloned katG gene (lanes 1, 3). Likewise, on introduction of the gene into E. coli significant levels of catalase-peroxidase were produced a striking increase in expression was obtained from the lacZ'-katG gene fusion which directed the synthesis of an 85 kD fusion protein (Fig. 9, lane 6).

The aim of the present study was to determine the nucleotide sequence of the katG gene and to use the information obtained to try and understand how its product mediates the INH-susceptibility of M. tuberculosis and, possibly, to explain the apparent instability of the katG

region of the genome. Repetitive DNA is often a source of chromosomal rearrangements and analysis of the DNA sequence upstream of katG revealed two copies of a 700 bp direct repeat. Since this element appears to be confined to this locus it is unlikely to serve as a target for an event, such as homologous recombination, which could lead to the deletion of the gene that is observed so frequently (Zhang et al. 1992, Nature; Zhang and Young 1993). Likewise, as a 70 kb stretch of the chromosome of M. tuberculosis H37Rv, encompassing katG, is devoid of copies of IS6110 and IS1081, these insertion sequences do not appear to be likely sources of instability. Rather, the presence of a cluster of major polymorphic tandem repeats, MPTR (Fig. 5; Hermans et al. 1992) situated upstream of katG, suggests that this might act as a recombinational hotspot. It may remove both the MPTR cluster and katG (Zhang and Young 1993). The availability of the sequence of the katG region will allow primers suitable for the polymerase chain reaction to be designed and thus facilitate studies aimed at both rapid detection of INH-resistance and understanding the molecular basis of chromosomal instability.

Perhaps the most intriguing feature of the M. tuberculosis catalase-peroxidase is its ability to mediate INH-susceptibility. In our current working hypothesis, the drug interacts with the enzyme and is converted by the peroxidase activity into a toxic derivative which acts at a second, as yet unknown, site (Zhang et al. 1992, Nature). Although horse radish peroxidase can effect this reaction (Pearson et al. 1988; Shoeb et al. 1985), and produce hydroxyl and organic free radicals, very few bacteria, including other mycobacteria, are sensitive to INH. This is intriguing as they contain genes homologous to katG (Fig. 7).

One explanation for this could be provided by the fact that most bacterial contain two catalases, one of which is a broad spectrum enzyme endowed with peroxidase activity, and that the second catalase, by preferentially removing H_2O_2, limits the ability of the catalase-peroxidase to oxidize INH. As M. tuberculosis lacks the latter activity its KatG enzyme can convert INH to the lethal form without competition for the electron acceptor.

Alternatively, there may be some unique features of the M. tuberculosis enzyme which promote toxicity or favor the interaction with the drug. Examination of the primary structures of the bacterial catalase-peroxidases was not instructive in this respect as they all share extensive sequence identities and contain two motifs characteristic of the active sites of peroxidases. Furthermore, it has been shown recently that expression of the E. coli katG gene can partially restore INH-susceptibility to drug-resistant mutants of M. tuberculosis suggesting that the endogenous enzyme may not possess any drug-specific properties (Zhang et al. 1993). Sequence comparison with the cytochrome c peroxidase from yeast has provided important information about the structural and functional organization of the KatG protein and led to the identification of the putatively-important catalytic residues (Fig. 8).

Now that the complete sequence of katG is available it will be possible to test some of these hypotheses by site-directed mutagenesis and to overproduce the enzyme so that detailed analysis of the enzymatic reaction, and its products, can be performed in vitro. Likewise, it should be a relatively simple matter to isolate mutants that have retained enzymatic activity but are unable to bind or oxidize INH. Of particular interest is the repetitive structure of

the enzyme and the prediction that the NH_2-terminal repeat contains the active site for peroxidases. This raises the possibility that katG genes, mutated, or truncated at the 3'-end, could arise. It is conceivable that their products, lacking the normal COOH-terminus which may be required for subunit-subunit interactions (Welinder 1991), would be unstable but still retain low enzyme activity. They would thus confer an intermediate level of INH-susceptibility, between that of $katG^+$ strains and mutants completely lacking the gene, as is often observed in clinical settings.

The invention may of course make use of a part of the above described 2.5 kb EcoRV-KpnI fragment, said part being nonetheless sufficiently long to provide for the selectivity of the in vitro detection of a Mycobacterium tuberculosis resistant to isoniazid.

The invention also relates to a kit for detecting multidrug resistant variants of M. tuberculosis wherein the kit comprises:

(a) a container means containing a probe for the gene encoding drug resistance; and

(b) a container means containing a control preparation of nucleic acid.

Needless to say that use can be made of any detection method alternative bringing into play the nucleodic sequence specific of nucleic acids of a Mycobacterium resistant to isoniazid, e.g. a method using an amplification technique and primers, whereby said primers may either be contained within said specific nucleotidic sequence, in order to provide for amplification fragments containing at least a part of the nucleotide sequence of the above mentioned probe, nonetheless sufficiently long to provide for the selectivity of the in vitro detection of a Mycobacterium tuberculosis resistant to

isoniazid, and finally detecting a possible mutation in any of the amplified sequences.

A preferred process alternative (oligotyping) for the detection of resistance to the selected antibiotic comprises:

- fragmenting the relevant gene or part thereof likely to carry the mutation into a plurality of fragments, such as by digestion of said relevant gene by selected restriction enzymes,
- hybridizing these fragments to complementary oligonucleotide probes, preferably a series of labelled probes recognizing under stringent conditions, all of the parts of the relevant gene of a corresponding control DNA of a strain non-resistant to the corresponding antibiotic,
- and relating the absence of hybridization of at least one of said oligonucleotide probes to any of the DNA fragments of the relevant gene of the mycobacterium under study as evidence of the presence of a mutation and, possibly, of a resistance to the corresponding antibiotic, particularly as compared to the runing of the test under the same conditions with the same oligonucleotides on the relevant gene(s) obtained from a strain (strains) not resistant to said antibiotic.

Another process alternative (SSCP analysis, i.e. analysis of Single Stranded Conformation Polymorphisms) comprises:

- digesting the DNA to be analyzed, particularly of the relevant gene,
- amplifying the fragments obtained, e.g. by PCR,
- recovering the amplified fragments, and
- separating them from one another according to sizes, e.g. by causing them to migrate, for instance on an electrophoretic gel,
- comparing the sizes of the different fragments with those

obtained from the DNA(s) of one or several control strains not resistant to the antibiotic, which had been subjected to a similar assay, and

- relating the polymorphism possibly detected to the existence of a mutation in the relevant gene, accordingly to a possible resistance to the corresponding antibiotic of the strain from which the DNA under study had been obtained.

Needless to say that any other method, including classical sequencing techniques, can resorted to for the achievement of the same purpose.

This method includes that known under the expression "oligotyping" for the detection of polymorphisms, reference is advantageously made to the method discloses by Orita et al. (reference was already made thereto herebefore) for the detection of polymorphisms based on the conformation of single strands.

The relevant gene in the case of resistance to isoniazid is of course the katG gene or a fragment thereof.

In the case of resistance to rifampicin, the relevant gene happens to be the rpoB gene which codes for the ßsub-unit of the RNA polymerases of said mycobacteria, or when only part of that gene is being used, preferably that part which includes the codons 400 to 450 of that rpoB gene.

Finally, in the case of resistance to steptomycin, the relevant gene contemplated is that of the rpsL gene that codes for the S12 protein of the small ribosome sub-unit or, when only part of said fragment is being used, preferably that part which includes the codon at the 43 position.

A preferred procedure, particularly in relation to the process alternative making use of PCR amplification is disclosed hereafter.

DNA is obtained from a biological sample (e.g. blood or

sputum) after removal of the cellular debris and lysis of the bacterial cells with an appropriate lysis buffer. PCR amplication can be carried out by classical methods, using a pair of primers, whose sequences are respectively complementary to fragments of each of the strands of the DNA to be amplified.

The procedure may be run further as follows:

- the amplification products (comprising e.g. from 100 to 300 nucleotides) are digested by means of suitable restriction endonuclease,
- the ADN strands obtained from the amplification medium are subjected to denaturation,
- the monostranded DNA strands are deposited on a neutral 5% polyacrylamid gel,
- the monostranded DNA strands are caused to migrate on said gel by means of electrophoresis,
- the DNA fragments that migrated on the polyacrilamid gel are transferred onto a nylon membrane according to a usual electrophoretic blotting technique and hybridized to labelled probes, for instance ^{32}P labelled probes, and
- the migration distances of the DNA fragments subjected to analysis are compared to those obtained from controls obtained under the same conditions of amplification, digestion, denaturation electrophoresis and transfer onto a nylon membrane, whereby said DNA had been obtained from an identical bacterial strain yet sensitive to the antibiotic under study.

For the production of the PCR primers as well as of the polygonucleotides probes used in the above disclosed "oligotyping" procedures, use is advantageously made of those complementary to the rpoB gene of wild M.tuberculosis inserted in a plasmid deposited under number I-12167 at the

CNCM on September 15, 1992.

The invention also relates more particularly to the nucleotidic sequence of a fragment of rpsL gene of Mycobacterium tuberculosis coding for the S12 protein of the small ribosome sub-unit, as well as to the nucleotidic sequence of a mutated rpsL gene fragment deemed responsible of the resistance to streptomycin.

By amplification of that nucleotidic sequence, the nucleotide sequence of the full rpsL gene can be obtained.

Further illustration of the invention will be provided in the following description of additional examples, having regard to the drawings in which:

- **figure 10** represents digrammatically the PCR strategy used for the study of different M.Leprae isolates, showing the coding sequence of rpoB sequence, whereby the sequenced regions are shown by hatched parts, and the position and reference of the amplification primers used being indicated on the upper line, whereas the sequencing primers are indicated bellow it;
- **figure 11** represents (A) the nucleotidic sequence of a short region of rpoB carrying the mutations that confer resistance to rifampicin with an indication of the changes of bases in the corresponding alleles and (B) a comparison between the aminoacids sequences of the domain I of region II of the β-sub-unit of the RNA polymerase of E.coli and M.Leprae, whereby the numbers of the residues and the differences in the mutated aminoacids have been indicated; the mutated aminoacid residues associated with rifampicin resistance as well as the frequency of its occurrences have been represented too,
- **figure 12** shows a complete sequence of the rpoB gene of M.Leprae,

- figure 13 represents the sequence of part of the rpoB gene of M.tuberculosis,
- figure 14 represents the sequence of a part of the rpsL gene of M.tuberculosis; both the sequence of the full rpsL gene of M.Leprae and that of its expression product, that is the S12 protein (whose starting aminoacid is noted by 1) are indicated. The positions of the ML51 and ML52 primers, as well as of the sequences of part of the rpsL gene of M.tuberculosis are provided belows those of M.Leprae. Only those positions which are different and the corresponding aminoacid changes are indicated.
- figure 15 represents the wild DNA sequence of the rpsL gene fragment coding for the S12 protein of the small ribosome sub-unit that is responsible for the resistance to streptomycin, as well as the corresponding aminoacid sequence of the S12 protein.

Example relative to the detection of rifampicin resistance of mycobacteria

The sensitivity to rifampicin has been determined in mice as disclosed by Grosset et al. (and Int. J. Lepr. 57:607-614). The cells of M.Leprae were obtained from mouse paws according to classic procedures. All resistant strains were able to grow in mice which received daily doses of 20 mg/Kg of rifampicin, whereas sensitive strains were killed at low rifampicin concentrations, less than 2 mg/Kg.

Relevant regions of the rpoB gene of extracted DNA was initiated upon using two pairs of biotinylated primers, whose sequences appear in the following table.

TABLE

Primer	Séquence
Brpo22	CAGGACGTCGAGGCGATCAC
rpo23	AACGACGACGTGGCCAGCGT
Brpo24	CAGACGGTGTTTATGGGCGA
rpo25	TCGGAGAAACCGAAACGCTC
rpo32	TCCTCGTCAGCGGTCAAGTA
rpo33	CTTCCCTATGATGACTG
rpo34	GGTGATCTGCTCACTGG
rpo35	GCCGCAGACGCTGATCA
rpo36	TTGACCGCTGACGAGGA
rpo37	GCCAGCGTCGATGGCCG

Upon using conventional techniques, amplification products comprising 310 and 710 bp were respectively obtained as shown in figure 1. The localization of the sequences of the different primers used in the table is also indicated on figure 10.

The DNAs obtained have been sequenced on the basis of the rpoB sequence of isolates sensitive to rifampicin. A plasmid containing the sequence of that gene has been deposited at CNCM on September 15, 1992 under number I-1266. Biotinylated PCR products were concentrated from the PCR reaction mixtures by contacting with streptavidin coated beads under agitation. The biotinylated strands attached to the beads were then recovered and sequenced. The sequences obtained were compared to the sequence of the rpoB gene of a wild type stain. Significant results were obtained as a result of sequencing of the wild gene (of a mycobacterium sensitive to rifampicin) and of corresponding sequences of the ß-sub-unit of four mutant strains resistant to rifampicin (**figure 11**).

Results were obtained starting from 102 strands obtained from patients infected with M.tuberculosis. Among this 102 strands 53 were sensitive to rifampicin and 49 resistant to rifampicin. The mutation was localized in the region 400-450 in 43 of the mutants and among the latter, the mutation occured in the region of ^{425}Ser into leu.

Example of detection of the resistance of mycobacteria to streptomycin

The culture of M.tuberbulosis strains and the test of their sensitivity to streptomycin have been carried out by the method of proportions on a Löwenstein-Ierva medium (Laboratory Method for Clinical Mycobacteriology - Hugo David - Véronique Lévy Frébault, M.F. Thorel, published by Institut

Pasteur).

The nucleotide sequence of the rpsL gene of M.Leprae led, by sequence analogy, to the construction of two primers, ML51 (CCCACCATTCAGCAGCTGGT) et ML52 (GTCGAGCGAACCGCGAATGA) suronnding regions including putative mutation sites liable of being responsible for the streptomycin resistance and suitable for the PCR reaction. The DNA of the used M.tuberculosis used as a matrix has enabled one to obtain a rpsL fragment of 306 pb. The nucleotide sequence of the sequenced fragments exhibited 28 differences with that of M.Leprae.

The rpsL genes or 43 strands of M.tuberculosis, among which 28 were resistant, have been amplified both by PCR and the SSCP technique.

DNA was extracted from 200 µl aliquots of M.tuberculosis samples (in average 10^4 to 10^5 bacteria) covered by 100µl of mineral oil by a congelation-decongelation technique (Woods and Cole, 1989 FEBS. Microbiol. Lett,65:305-308).

After electrophoresis of the DNA strands tested a mutation was shown in 16 of the mutants. In order to establish the nature of the mutation in the 16 strands under consideration, the corresponding rpsL gene fragments were amplified by PCR using the ML51 and the ML52 primers and their respective nucleotide sequences were determined.

The sequences obtained were compared to the sequence of the wild type rpsL gene. The single difference was found with the wild sequence ; codon 43, AAG, was mutated into AGG and, consequently, the lys-42 aminoacid was replaced by Arg.

The invention relates also to the "mutated" DNA fragments. They can in turn be used as hybridization probes for use for the dectection in suitable hybridization

procedures and for the detection of similar mutation in DNA extracted from a M.tuberbulosis strain suspected to include resistance to any one of the above illustrated antibiotics.

The invention further relates to kits for the resistance of mycobacteriae to isoniazid, rifampicin or analogues thereof, and streptomycin.

The invention further relates to a kit for the in vitro diagnostic of the resistance of a bacteria of a mycobacterium genus to isoniazid, characterized in that it comprises:

- means for carrying out for a genic amplification of the DNA of the katG gene or of a fragment thereof,
- means to bring into evidence one or several mutations on the amplification products so obtained,
- a preparation of control DNA of a katG gene of a strain of said bacteria sensitive to isoniazid or of a fragment thereof,
- optionally, a control preparation of a DNA of the katG gene of an isoniazid-resistant mycobacterium strain.

The invention further relates to a kit for the in vitro diagnostic of the resistance of a bacteria of a mycobacterium genus to rifampicin or its analogues, characterized in that it comprises:

- means for carrying out for a genic amplification of the DNA of the rpoB gene or of the ß-sub-unit of the RNA polymerase of said mycobacteria, or of a fragment thereof,
- means to bring into evidence one or several mutations on the amplification products so obtained,
- a preparation of control DNA of a rpoB gene coding for the ß-sub-unit of the RNA polymerase of a strain of said bacteria sensitive to rifampicin or of a fragment thereof,
- optionally, a control preparation of a DNA of the rpoB gene of an isoniazid-resistant mycobacterium strain.

Similarly, the invention pertains to a kit for the *in vitro* diagnostics of the resistance of the *M.tuberculosis* to streptomycin, characterized in that it includes:

- means for carrying out a genic amplification of the *rpsL* gene coding for the S12 protein of the small ribosome sub-unit, or fragment thereof,
- means which enable the bringing to evidence of one or several mutations on the amplification products obtained,
- a control preparation of a DNA sequence of the *rpsL* gene coding for the S12 protein of the small sub-unit of the ribosome of a *M.tuberculosis* strain sensitive to streptomycin, and
- optionally, a control preparation of a DNA sequence of a *rpsL* gene coding for the S12 protein of the small sub-unit of the ribosome of a strain of *M.tuberculosis* resis tant to streptomycin.

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CLAIMS

WHAT IS CLAIMED IS:

1. A process for the detection of a resistance to an antibiotic in a mycobacterium which comprises detecting a mutation in a gene selected from the group comprising the katG gene or fragment thereof, the rpoB gene or fragment thereof and the rpsL gene or fragment thereof.

2. A process of claim 1 for detecting in vitro the presence of nucleic acids of a Mycobacterium tuberculosis resistant to isoniazid, wherein the process comprises the steps of:

- contacting said nucleic acids previously made accessible to a probe if required under conditions permitting hybridization;
- detecting any probe that had hybridized to said nucleic acids;

wherein said probe comprises a nucleic acid sequence, which is 2.5 kb EcoRV-KpnI fragment of plasmid pYZ56 or of part thereof, and wherein said fragment contains a BamHI cleavage site, wherein said part is nonetheless sufficiently long to provide for the selectivity of the in vitro detection of a Mycobacterium tuberculosis resistant to isoniazid.

3. The process as claimed in claim 2, which comprises the steps of :

(A) depositing and fixing nucleic acids of Mycobacterium tuberculosis on a solid support, so as to make the nucleic acids accessible to a probe;

(B) contacting said fixed nucleic acids from step (A) with the probe under conditions permitting hybridization;

(C) washing said filter resulting from step (B), so as to eliminate any non-hybridized probe; and then

(D) detecting any hybridized probe on said washed filter resulting from step (C);

4. The process of claim 2 or 3 wherein said probe comprises a nucleic acid sequence which encodes a polypeptide of the formula APLNSWPDNASLDKAR-RLLWPSKKKYGKKLSWADLIV.

5. A process as claimed in any of claims 2 to 4, wherein the probe has a radioactive label selected from the group consisting of radioactive, enzymatic, fluorescent, and luminescent labels.

6. The use of the process of any one of claims 2 to 5 for the detection of the presence of Mycobacterium tuberculosis resistant to isoniazid in a bacteria-containing sample suspected of containing Mycobacterium tuberculosis resistant to isoniazid, whereby the detection of the probe that had hybridized, particularly in the form of a hybrid DNA complex that it either forms or had formed with DNA initially present in said sample, is indicative of the presence in said sample of Mycobacterium tuberculosis resistant to isoniazid.

7. The use of claim 6, wherein prior to the contacting of said DNA with said probe, said bacteria had been separated from said sample and immobilized on a DNA binding support, such as a nitrocellulose membrane.

8. A kit for the detection of Mycobacterium tuberculosis resistant to isoniazid, wherein the kit comprises:

(A) a container means containing a probe, preferably labelled by a label selected from the group consisting of radioactive, enzymatic, fluorescent, and luminescent labels, comprising a nucleic acid sequence, which is a 2.5 kb EcoRV-KpnI fragment of plasmid pYZ56 or part thereof, wherein said fragment contains a BamHI cleavage site and

nonetheless sufficiently long to provide for the selectivity of the in vitro detection of a Mycobacterium tuberculosis resistant to isoniazid; and

(B) a container means containing a control preparation of nucleic acid.

9. A nucleic acid probe for detecting Mycobacterium tuberculosis resistant to isoniazid, wherein said probe consists of a 2.5 kb EcoRV-KpnI fragment of plasmid pYZ56, wherein said fragment contains a BamHI cleavage site, or of a part of said fragment nonetheless sufficiently long to provide for the selectivity of the in vitro detection of a Mycobacterium tuberculosis resistant to isoniazid.

10. The probe as claimed in claim 9, which is DNA free of human serum proteins or human tissue or both, viral proteins, bacterial proteins, and nucleotide sequences encoding said proteins.

11. A hybrid duplex molecule consisting essentially of the probe of claim 9 hydrogen bonded to a nucleotide sequence of complementary base sequence.

12. A process for selecting a nucleotide sequence of a Mycobacterium tuberculosis resistant to isoniazid from a group of nucleotide sequences, comprising the step of determining which of said nucleotide sequences hybridizes to a probe as claimed in claim 9 or 10.

13. A process for selecting a compound active against Mycobacterium tuberculosis comprising the step of determining the reactivity of the compound on INH-resistant Mycobacterial strains.

14. A nucleotide sequence comprising the 350 base sequence or a portion thereof as described in Figure 2.

15. A process for detecting point mutations or partial

deletion of the KatG gene comprising contacting a sample of Mycobacterium tuberculosis with the probe of claim 9 or 10.

16. The process of claim 1 for the detection of resistance to the selected antibiotic which comprises:

- fragmenting the relevant gene or part thereof likely to carry the mutation into a plurality of fragments, such as by digestion of said relevant gene by selected restriction enzymes,
- hybridizing these fragments to complementary oligonucleotide probes, preferably a series of labelled probes recognizing under stringent conditions, all of the parts of the relevant gene of a corresponding control DNA of a strain non-resistant to the corresponding antibiotic,
- and relating the absence of hybridization of at least one of said oligonucleotide probes to any of the DNA fragments of the relevant gene of the mycobacterium under study as evidence of the presence of a mutation and, possibly, of a resistance to the corresponding antibiotic, particularly as compared to results obtained upon running the test under the same conditions with the same oligonucleotides on the relevant gene(s) obtained from a strain (strains) not resistant to said antibiotic, wherein said relevant gene is either the katG gene or a fragment thereof, the rboB gene or a fragment thereof, the rpsL gene or a fragment thereof.

17. The process of claim 1 which comprises:

- digesting the DNA to be analyzed, particularly of the relevant gene,
- amplifying the fragments obtained, e.g. by PCR,
- recovering the amplified fragments, and
- separating them from one another according to sizes, e.g. by causing them to migrate, for instance on an electrophoretic gel,

- comparing the sizes of the different fragments with those obtained from the DNA(s) of one or several control strains not resistant to the antibiotic, which had been subjected to a similar assay, and
- relating the polymorphism possibly detected to the existence of a mutation in the relevant gene, accordingly to a possible resistance to the corresponding antibiotic of the strain from which the DNA under study had been obtained, wherein said relevant gene is either the katG gene or a fragment thereof, the rboB gene or a fragment thereof, the rpsL gene or a fragment thereof.

18. A kit for the in vitro diagnostic of the resistance of a bacteria of a mycobacterium genus to isoniazid, characterized in that it comprises:
- means for carrying out for a genic amplification of the DNA of the katG gene or of a fragment thereof,
- means to bring into evidence one or several mutations on the amplification products so obtained,
- a preparation of control DNA of a katG gene of a strain of said bacteria sensitive to isoniazid or of a fragment thereof,
- optionally, a control preparation of a DNA of the katG gene of an isoniazid-resistant mycobacterium strain.

19. A kit for the in vitro diagnostic of the resistance of a bacteria of a mycobacterium genus to rifampicin or its analogues, characterized in that it comprises:
- means for carrying out for a genic amplification of the DNA of the rpoB gene or of the ß-sub-unit of the RNA polymerase of said mycobacteria, or of a fragment thereof,
- means to bring into evidence one or several mutations on the amplification products so obtained,
- a preparation of control DNA of a rpoB gene coding for the

ß-sub-unit of the RNA polymerase of a strain of said bacteria sensitive to rifampicin or of a fragment thereof,

- optionally, a control preparation of a DNA of the rpoB gene of an isoniazid-resistant mycobacterium strain.

20. A kit for the in vitro diagnostics of the resistance of the M.tuberculosis to streptomycin, characterized in that it includes:

- means for carrying out a genic amplification of the rpsL gene coding for the S12 protein of the small ribosome sub-unit, or fragment thereof,
- means which enable the bringing to evidence of one or several mutations on the amplification products obtained,
- a control preparation of a DNA sequence of the rpsL gene coding for the S12 protein of the small sub-unit of the ribosome of a M.tuberculosis strain sensitive to streptomycin, and
- optionally, a control preparation of a DNA sequence of a rpsL gene coding for the S12 protein of the small sub-unit of the ribosome of a strain of M.tuberculosis resistant to streptomycin.

FIG. 1

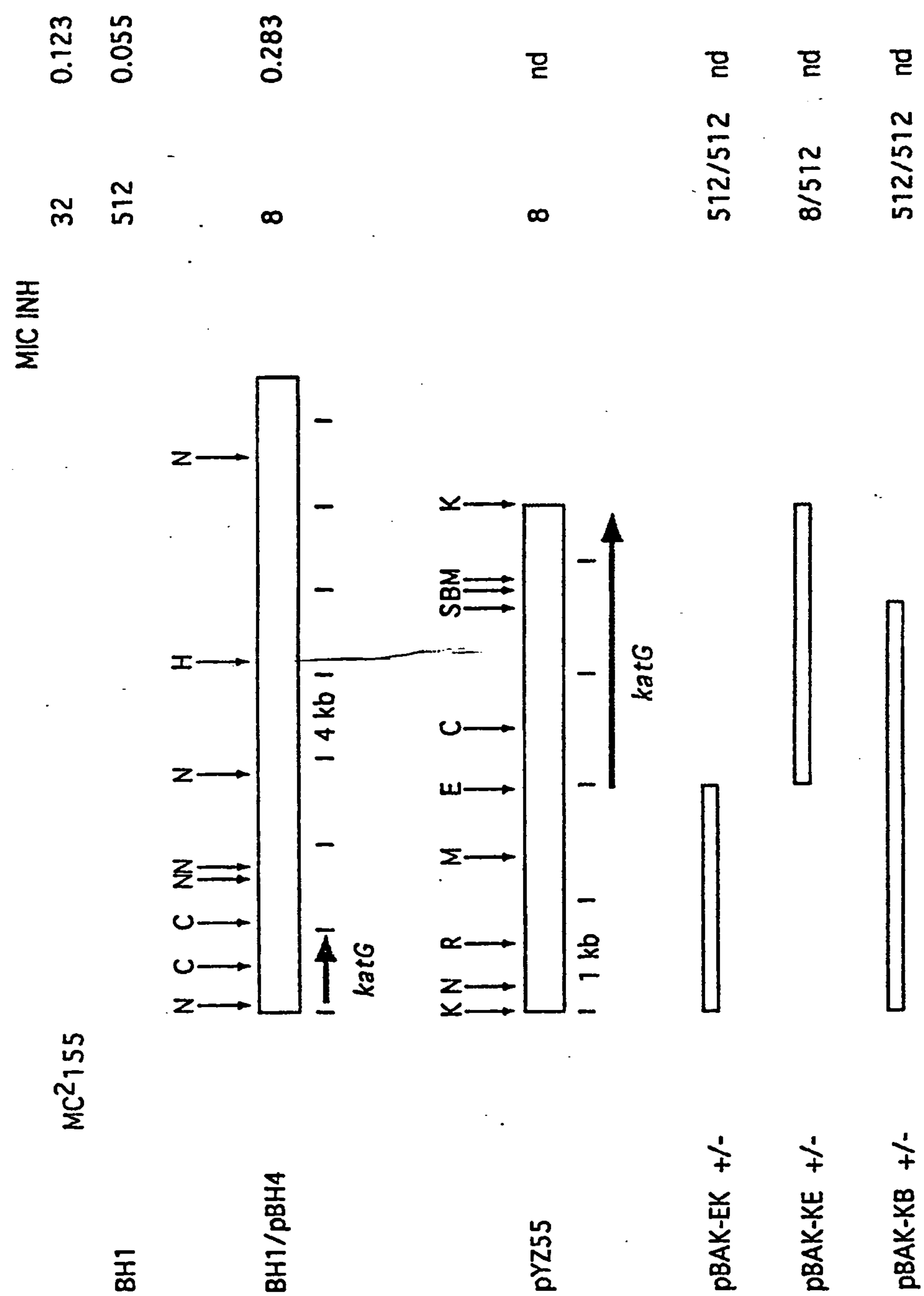

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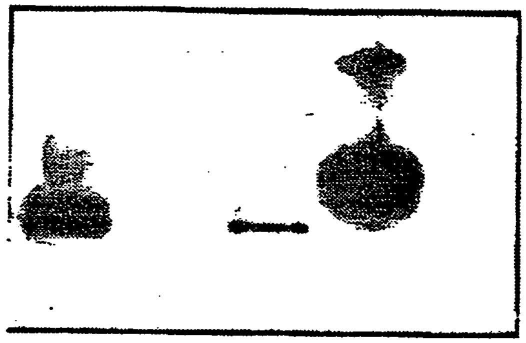
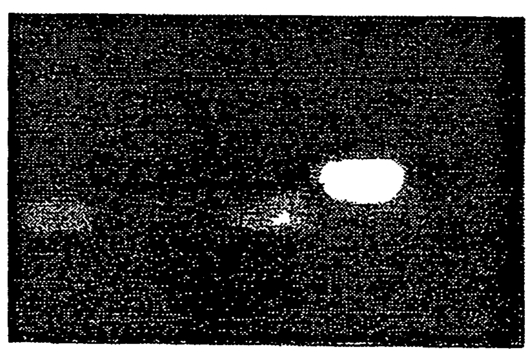
FIG. 2A



1 2 3 4 5

FIG. 2B



1 2 3 4 5

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FIG. 2C(I)

```
<------------------lacZ'---------------------------->

 M  T  M  I  T  P  S  L  H  A  C  R  S  T  L  E  D  P  H  P  T  L  R
ATGACCATGATTACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCATCCGACACTTCGCG
         10        20        30        40        50        60        70

katG------->

 M  S  T  S  D  D  -  -  I  H  N  T  T  A  T  G  K  C  P  F  H  Q  G
 *  :                             *  *  :  :  :        :
 M  P  E  Q  H  P  P  I  T  E  T  T  T  G  A  A  S  N  G  C  P  V  V
GTGCCCGAGCAACACCCACCCATTACAGAAACCACCACCGGAGCCGCTAGCAACGGCTGTCCCGTCGTGG
        130       140       150       160       170       180       190

 N  Q  L  R  V  D  L  L  N  Q  H  S  N  R  S  N  P  L  G  E  D  F  D
 *     *     :     :  *     *     :        :  :  *  :  *        *  *
 N  R  L  N  L  K  V  L  H  Q  N  P  A  V  A  D  P  M  G  A  A  F  D
AACCGGCTCAATCTGAAGGTACTGCACCAAAACCCGGCCGTCGCTGACCCGATGGGTGCGGCGTTCGACT
        250       260       270       280       290       300       310
```

FIG. 2C(2)

```
D H I R D H S P I T P T P G R N A
ATCACATCCGTGATCACAGCCCCGATAACACCAACTCCTGGAAGGAATGCT
        80        90       100       110       120

G H D Q S A G A G T T T R D W W P  E.coli
* *       :     * : :     * * * *
G H M K Y P V E G G G N Q D W W P  M.tub
GTCATATGAAATACCCCCGTCGAGGGCGGCGGAAACCAGGACTGGTGGCCC
       200       210       220       230       240

Y R K E F S K L D Y Y G L K K D L  E.coli
*     * : :               : *   : * :
Y A A E V A T S R L D A L T R D I  M.tub
ATGCCGCGGAGGTCGCGACCAGTCGACTTGACGCCCTGACGCGGGACATC
       320       330       340       350       360
```

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FIG. 3

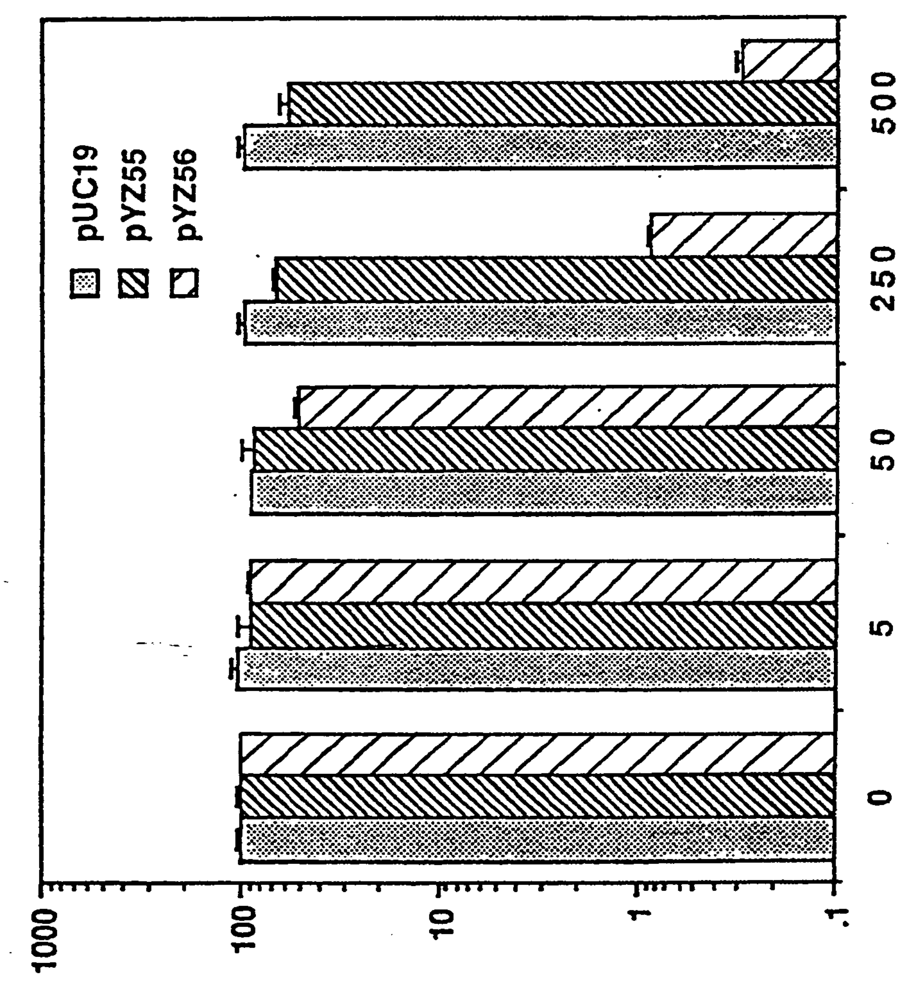


FIG. 4A

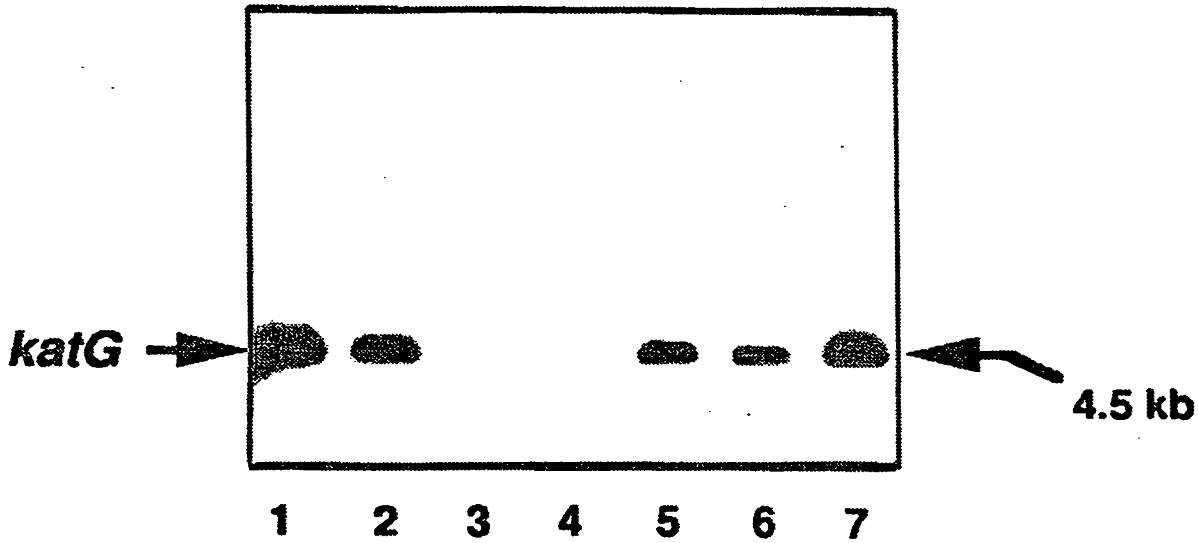
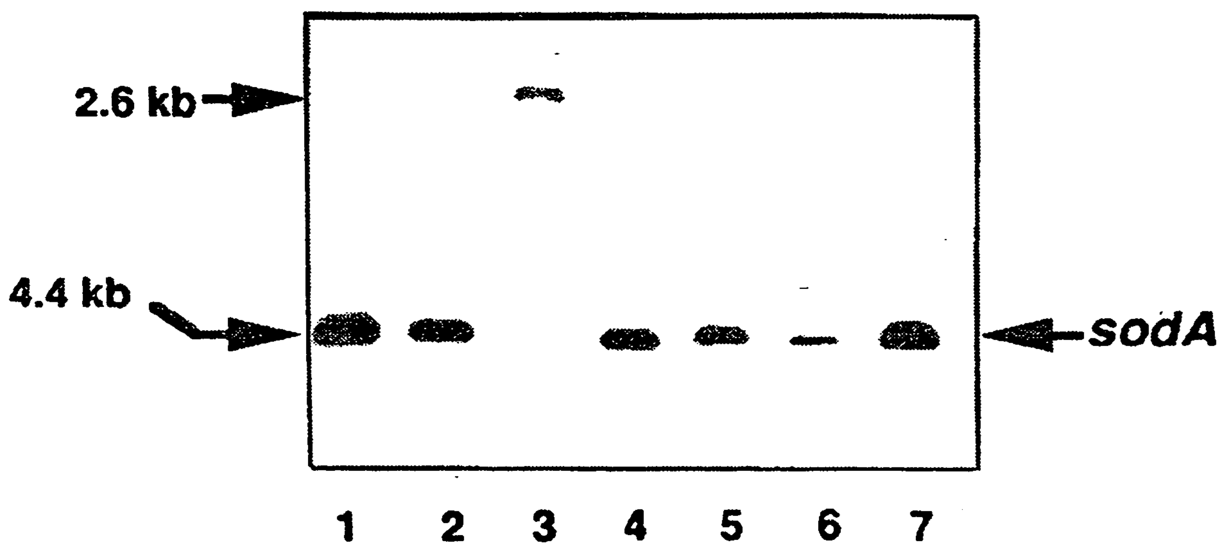


FIG. 4B



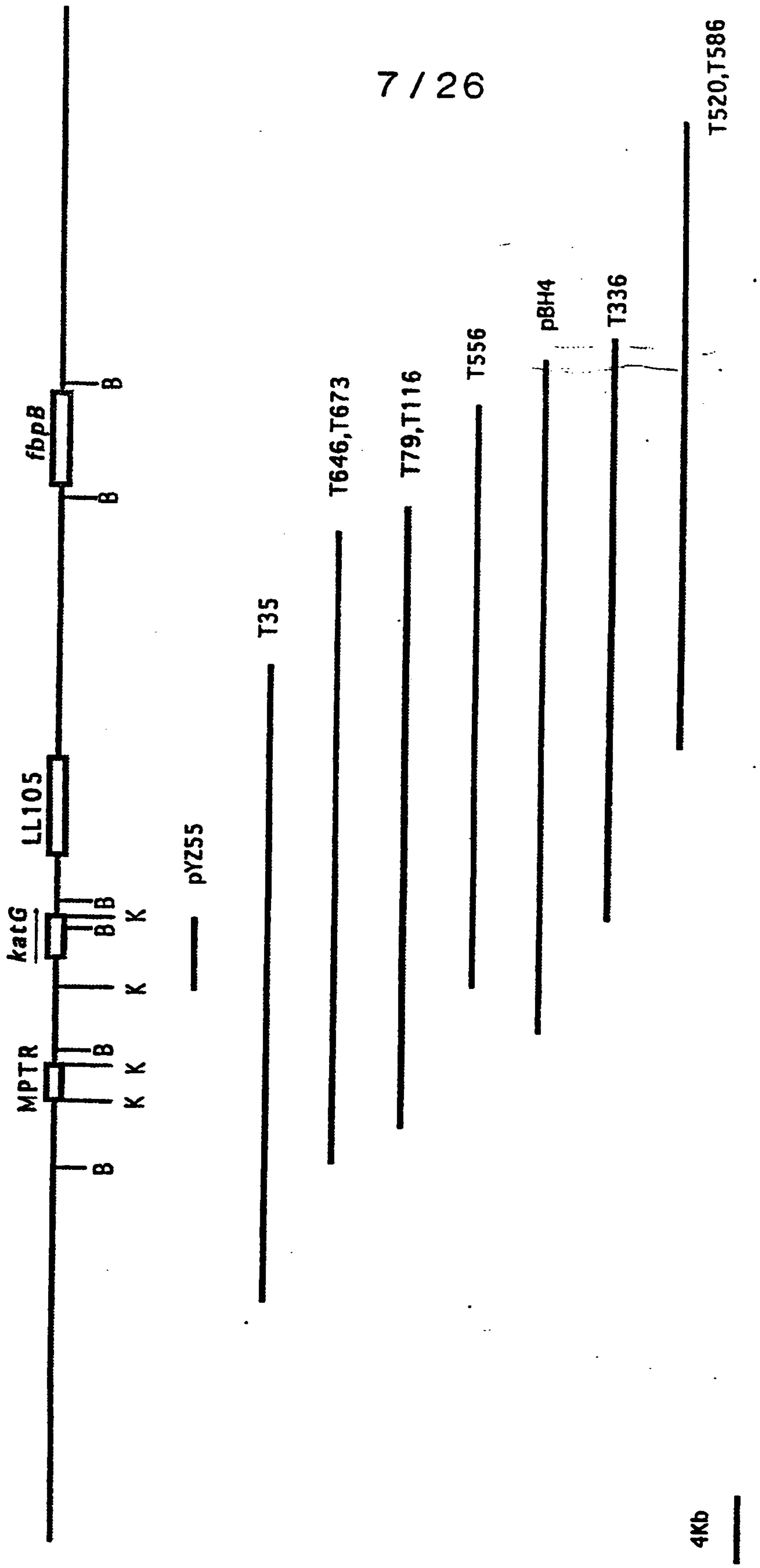

fbpB
B
B
LL105
katG
B
B
K
K
MPTR
B
K
K
B
pYZ55
T35
T646,T673
T79,T116
T556
pBH4
T336
T520,T586
4Kb

FIG. 5

FIGURE 6A(1)

```
GGTACCGTGA GGCGATGGGT GGCCCGGGGC CCGGCTGTCT GGTAAGCGCG GCCGCAAAAC     60
AGCTGTACTC TCGAATCCCA GTTAGTAACA ATGTGCTATG GAATCTCCAA TGACGAGCAC    120
ACTTCACCGA ACCCCATTAG CCACCGCGGG GCTGGCGCTC GTAGTGGCGC TGGGTGGCTG    180
CGGGGGCGGG GGCGGTGACA GTCGAGAGAC ACCGCCATAC GTGCCGAAAG CGACGACCGT    240
CGACGCAACA ACGCCGGCGC CGGCCGCCGA GCCACTGACG ATCGCCAGTC CCATGTTCGC    300
CGACGGCGCC CCGATCCCGG TGCAATTCAG CTGCAAGGGG GCCAACGTGG CCGCCACCGT    360
TGACGTGGTC GTCGCCCGCG GCGAGCGAAC TGGCACTCGT CGTCGATGAC CCCGACGCGG    420
TCGGCGGACT GTACGTGCAC TGGATCGTGA CCGGAATCGC CCCTGGCTCT GGCAGCACGG    480
CGGATGGTCA GACTCCTGCT GGTGGGCACA GCGTGCCGAA TTCTGGTGGT CGGCAAGGAT    540
ACTTCGGTCC ATGCCCGCCG GCGGGCACCG GGACACACCA CTACCGGTTT ACCCTCTACC    600
ACCTTCCTGT CGCGCTCCAG CTGCCACCGG GAGCCACGGG AGTCCAAGCG GCACAGGCGA    660
TAGCACAGGC CGCCAGCGAC AGGCCCGGCT CGTCGGCACA TTCGAAGGCT GACGCCGCGG    720
CATCCCTGGC GAGGTGGTCG AAACCCTGGC TTCTCCAATT GCGCCTGGCG ACAATGATCA    780
ATATGGAATC GACAGTGGCG CACGCATTTC ACCGGTTCGC ACTGGCCATC TTGGGGCTGG    840
CGCTCCCCGT GGCGCTAGTT GCCTACGGTG GCAACGGTGA CAGTCGAAAG GCGGCGGCCG    900
TGGCGCCGAA AGCAGCAGCG CTCGGTCGGA GTATGCCCGA AACGCCTACC GGCGATGTAC    960
TGACAATCAG CAGTCCGGCA TTCGCCGACG GTGCGCCGAT CCCGGAACAG TACACCTGCA   1020
AAGGAGCCAA TATCGCGGCC TCCGTTGACC TGGTCGGCGC CGTTTGGCGG CGCACTCGTT   1080
GTCGATGATC CGGACCACCT CGCGAACCTT ACGTCCATTG GATCGTGATC GGGATCGCCC   1140
CTGGTGCTGG CAGCAGCCGA TGGTGAGACT CCCGGTGGCG GAATCAGCCT GCCGAACTCC   1200
AGCGGTCAGC CCGCATACAC CGGCCCCTGC CCGCCGGCGG GCACCGGGAC ACACCACTAC   1260
```

FIGURE 6A(2)

```
CGGTTTACCC TCTACCACCT TCCTGCCGTG CCTCCACTCG CGGGACTGGC TGGGACACAA   1320
GCGGCGCGGG TGATCGCGCA GGCCGCCACC ATGCAGGCCC GGCTCATCGG AACATACGAA   1380
GGCTGATCCA CCCGCCATCC CACGATCCAG CGGCCCCGGG CGATCGGGTC CTAGCAGACG   1440
CCTGTCACGC TAGCCAAAGT CTTGACTGAT TCCAGAAAAG GGAGTCATAT TGTCTAGTGT   1500
GTCCTCTATA CCGGACTACG CCGAACAGCT CCGGACGGCC GACCTGCGCG TGACCCGACC   1560
GCGCGTCGCC GTCCTGGAAG CAGTGAATGC GCATCCACAC GCCGACACGG AAACGATTTT   1620
CGGTGCCGTG CGTTTTGCGC TGCCCGACGT ATCCGGCAAG CCGTGTACGA CGTGCTGCAT   1680
GCCCTGACCG CCGCGGGCTT GGTGCGAAAG ATCCAACCCT CGGGCTCCGT CGCGCGCTAC   1740
GAGTCCAGGG TCGGCGACAA CCACCATCAC ATCGTCTGCC GGTCTTGCGG GGTTATCGCC   1800
GATGTCGACT GTGCTGTTGG CGAGGCACCC TGTCTGACGG CCTCGGACCA TAACGGCTTC   1860
CTGTTGGACG AGGCGGAGGT CATCTACTGG GGTCTATGTC CTGATTGTTC GATATCCGAC   1920
ACTTCGCGAT CACATCCGTG ATCACAGCCC GATAACACCA ACTCCTGGAA GGAATGCTGT   1980
GCCCGAGCAA CACCCACCCA TTACAGAAAC CACCACCGGA GCCGCTAGCA ACGGCTGTCC   2040
CGTCGTGGGT CATATGAAAT ACCCCGTCGA GGGCGGCGGA AACCAGGACT GGTGGCCCAA   2100
CCGGCTCAAT CTGAAGGTAC TGCACCAAAA CCCGGCCGTC GCTGACCCGA TGGGTGCGGC   2160
GTTCGACTAT GCCGCGGAGG TCGCGACCAG TCGACTTGAC GCCCTGACGC GGGACATCGA   2220
GGAAGTGATG ACCACCTCGC AGCCGTGGTG GCCCGCCGAC TACGGCCACT ACGGGCCGCT   2280
GTTTATCCGG ATGGCGTGGC ACGCTGCCGG CACCTACCGC ATCCACGACG GCCGCGGCGG   2340
CGCCGGGGGC GGCATGCAGC GGTTCGCGCC GCTTAACAGC TGGCCCGACA ACGCCAGCTT   2400
GGACAAGGCG CGCCGGCTGC TGTGGCCGGT CAAGAAGAAG TACGGCAAGA AGCTCTCATG   2460
GGCGGACCTG ATTGTTTTCG CCGGCAACCG CTGCGCTCGG AATCGATGGG CTTCAAGACG   2520
TTCGGGTTCG GCTTCGGGCG TCGACCAGTG GGAGACCGAT GAGGTCTATT GGGGCAAGGA   2580
```

FIGURE 6A(3)

```
AGCCACCTGG CTCGGCGATG ACGGTTACAG CGTAAGCGAT CTGGAGAACC CGCTGGCCGC   2640
GGTGCAGATG GGGCTGATCT ACGTGAACCC GGAGGCGCCG AACGGCAACC CGGACCCCAT   2700
GGCCGCGGCG GTCGACATTC GCGAGACGTT TCGGCGCATG GCCATGAACG ACGTCGAAAC   2760
AGCGGCGCTG ATCGTCGGCG GTCACACTTT CGGTAAGACC CATGGCGCCG GCCCGGCCGA   2820
TCTGGTCGGC CCCGAACCCG AGGCTGCTCC GCTGGAGCAG ATGGGCTTGG GCTGGAAGAG   2880
CTCGTATGGC ACCGGAACCG GTAAGGACGC GATCACCAGC GGCATCGAGG TCGTATGGAC   2940
GAACACCCCG ACGAAATGGG ACAACAGTTT CCTCGAGATC CTGTACGGCT ACGAGTGGGA   3000
GCTGACGAAG AGCCCTGCTG GCGCTTGGCA ATACACCGCC AAGGACGGCG CCGGTGCCGG   3060
CACCATCCCG GACCCGTTCG GCGGGCCAGG GCGCTCCCCG ACGATGCTGG CCACTGACCT   3120
CTCGCTGCGG GTGGATCCGA TCTATGAGCG GATCACGCGT CGCTGGCTGG AACACCCCGA   3180
GGAATTGGCC GACGAGTTCC GCAAGGCCTG GTACAAGCTG ATCCACCGAG ACATGGGTCC   3240
CGTTGCGAGA TACCTTGGGC CGCTGGTCCC CAAGCAGACC CTGCTGTGGC AGGATCCGGT   3300
CCCTGCGGTC AGCACGACCT CGTCGGCGAA GCAGATTGCC AGCCTTAAGA GCCAGATCCG   3360
GGCATCGGGA TTGACTGTCT CACAGCTAGT TTCGACCGCA TGGGCGGCGG CGTCGTCGTT   3420
CCGTGGTAGC GACAAGCGCG GCGGCGCCAA CGGTGGTCGC ATCCGCCTGC AGCCACAAGT   3480
CGGGTGGGAG GTCAACGACC CCGACGGATC TGCGCAAGGT CATTCGCACC CTGAAGAGAT   3540
CCAGGAGTCA TTCACTCGGC GCGGGAACAT CAAAGTGTCC TTCGCCGACC TCGTCGTGCT   3600
CGGTGGCTGT GCGCCACTAG AGAAAGCAGC AAAGGCGGCT GGCCACAACA TCACGGTGCC   3660
CTTCACCCCG GGCCCGCACG ATGCGTCGCA GGAACAAACC GACGTGGAAT CCTTTGCCGT   3720
GCTGGAGCCC AAGGCAGATG GCTTCCGAAA CTACCTCGGA AAGGGCAACC GTTGCCGGCC   3780
GAGTACATCG CTGCTCGACA AGGCGAACCT GCTTACGCTC AGTGCCCCTG AGATGACGGT   3840
GCTGGTAGGT GGCCTGCGCG TCCTCGGCGC AAACTACAAG CGCTTACCGC TGGGCGTGTT   3900
```

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FIGURE 6A(4)

```
CACCGAGGCC TCCGAGTCAC TGACCAACGA CTTCTTCGTG AACCTGCTCG ACATGGGTAT  3960
CACCTGGGAG CCCTCGCCAG CAGATGACGG GACCTACCAG GGCAAGGATG GCAGTGGCAA  4020
GGTGAAGTGG ACCGGCAGCC GCGTGGACCT GGTCTTCGGG TCCAACTCGG AGTTGCGGGC  4080
GCTTGTCGAG GTCTATGCGC CGATGACGCG GCAGGCGAAG TTCGTGACAG GATTCGTCGC  4140
TGCGTGGGAC AAGGTGATGA ACCTCGACAG GTTCGACGTG CGCTGATTCG GGTTGATCGG  4200
CCCTGCCCGC CGATCAACCA CAACCCGCCG CAGCACCCCG CGAGCTGACC GGCTCGCGGG  4260
GTGCTGGTGT TTGCCCGGCG CGATTTGTCA GACCCCGCGT GCATGGTGGT CGCACGGACG  4320
CACGAGACGG GGATGACGAG ACGGGGATGA GGAGAAAGGG CGCCGAAATG TGCTGGATGT  4380
GCGATCACCC GGAAGCCACC GCCGAGGAGT ACCTCGACGA GGTGTACGGG ATAATGCTCA  4440
TGCATGGCTG GGCGGTACAG CACGTGGAGT GCGAGCGACG GCCATTTGCC TACACGGTTG  4500
GTCTAACCCG GCGCGGCTTG CCCGAACTGG TGGTGACTGG CCTCTCGCCA CGACGTGGGC  4560
AGCGGTTGTT GAACATGCCG TCGAGGGCTC TGGTCGGTGA CTTGCTGACT CCCGGTATGT  4620
AGACCACCCT CAAAGCCGGC CCTCTTGTCG AAACGGTCCA GGCTACACAT CCGGACGCGC  4680
ATTTGTATTG TGCGATCGCC ATCTTTGCGC ACAAGGTGAC GGCCTTGCAG TTGGTGTGGG  4740
CCGACCGCGT GGTCGCTGGC CGTGGGCGGC GGACTTCGAC GAAGGTCGCG GTACC       4795
```

FIGURE 6B(1)

```
   --T----T-- --CGAAGGCT GACGCCG--- --CGGCATCC CTGGCGAG-G TGGTCG-AAA
     *    *      *** ** * * * ***     **** *   ****  ** *  ** ** ***
   GGTACCGTGA GGCGATGGGT GGC-CCGGGG CCCGGC-TGT CTGGTAAGCG CGGCCGCAAA
   1          11         21         31         41         51
761        771        781        791        801        811
   CC--CTGG-C TTCTCCAATT GCGCCTG--- GCGACAATGA T-CAATATGG AATCGACAGT
    *  ***   * * *** ***   * ** *    *  ******  * *  ***** ****  ** *

   ACAGCTGTAC T-CTCGAAT- -C-CCAGTTA GTAACAATG- TGC--TATGG AATCTCCAAT
   61         71         81         91         101        111
821        831        841        851        861        871
   GGCG--CACG CATTTCACCG GTTCGCACTG GCCATCTTGG GGCTGGCGCT CCCCGTGGCG
   * **  ***   * *******    * ** *  **** *  ** ********** *   ******
   GACGAGCAC- -ACTTCACCG AACCCCATTA GCCACCGCGG GGCTGGCGCT CGTAGTGGCG
   121        131        141        151        161        171
881        891        901        911        921        931
   CTAGTTGCCT ACGGTGGCAA ---CGGTGAC AGTCGAAAGG CGGCGGC--- CGTGGCGCCG
   ** * ** **  *** ***      ******* ****** **   *  ** *   ***  ****
   CTGGGTGGCT GCGGGGGCGG GGGCGGTGAC AGTCGAGAGA CACCGCCATA CGT---GCCG
   181        191        201        211        221        231
941        951        961        971        981        991
   AAAGCAGCAG CG-CTCGGTC G--G--AGTA TGCCCGAAAC GCCTACCGGC GATGT-ACTG
   ***** * *  ** * ** ** *  *  *  *  *** *    * ***  *** * ** *  ****
   AAAGC-G-A- CGAC-CG-TC GACGCAACAA CGCCGG---C GCCGGCCGCC GA-GCCACTG
   241        251        261        271        281        291
1001       1011       1021       1031       1041       1051
   ACAATCAGC- AGTCCGGCAT --TCGCCGAC GGTGCGCCGA TCCCGGAACA GTACACCTGC
   ** *** **  *****  ***   ******** ** ** **** ******  **  * ** ****
   ACGATC-GCC AGTCC--CAT GTTCGCCGAC GGCGCCCCGA TCCCGGTGCA ATTCAGCTGC
   301        311        321        331        341        351
1061       1071       1081       1091       1101       1111
   AAAGGAGCCA ATATCGCGGC CTCCGTTGAC CTGGTCGGCG CC-GTTTGGC G-GCG-----
   ** ** **** *  * ** ** * ********  ****** ** ** *    *** * ***
   AAGGGGGCCA ACGTGGCCGC CACCGTTGAC GTGGTCGTCG CCCGC--GGC GAGCGAACTG
   361        371        381        391        401        411
1121       1131       1141       1151       1161       1171
   -CACTCGTTG TCGATGATCC GGAC-CACCT CG-CGAACCT -TACGTCCAT TGGATCGTGA
    ******* * ******* **   *** *    * ** ** ** *  ***** **  **********

   GCACTCGTCG TCGATGACCC CGACGCGG-T CGGCGGAC-T GTACGTGCAC TGGATCGTGA
   421        431        441        451        461        471
1181       1191       1201       1211       1221       1231
   TCGGGATCGC CCCTGG-TGC TGGCAGCA-- GCCGATGGTG AGACTCCCGG TGGCGGA-AT
    *** ***** ****** * * ********    ** ******  ******* *  *** **  *
   CCGGAATCGC CCCTGGCT-C TGGCAGCACG GCGGATGGTC AGACTCCTGC TGGTGGGCA-
481        491        501        511        521        531
```

FIGURE 6B(2)

```
1241      1251       1261       1271       1281       1291
 CAGCCTGCCG AACTCCAGCG GTCAGCCCGC ATACACCGGC CCCTGCCCGC CGGCGGGCAC
 **** ***** ** **   * * *** **   *  ****   ***  ** ******* **********
 CAGCGTGCCG AATTCTGGTG GTCGGCAAGG ATACTTCGGT CCATGCCCGC CGGCGGGCAC
541       551        561        571        581        591
1301      1311       1321       1331       1341       1351
 CGGGACACAC CACTACCGGT TTACCCTCTA CCACCTTCCT GCCGTGCCTC CA-CTCGC--
 ********** ********** ********** ********** * ** ** ** ** ** **
 CGGGACACAC CACTACCGGT TTACCCTCTA CCACCTTCCT GTCGCGC-TC CAGCT-GCCA
601       611        621        631        641        651
1361      1371       1381       1391       1401       1411
 --GGGACTGG CT--GGGA-- CACAAGCGGC GCGGGTGATC GCGCAGGCCG CCACCATG-C
   ****   * *   ****   * ********  * ** ***  ** ******* *** *   * *
 CCGGGA---G CCACGGGAGT C-CAAGCGGC ACAGGCGATA GCACAGGCCG CCAGC--GAC
661       671        681        691        701        711
1421      1431       1441       1451       1461
 AGGCCCGGCT CATCGGAACA TACGAAGGCT GATCCACCCG CCATCC
 ********** * **** ***
 AGGCCCGGCT CGTCGGCACA ---------- ---------- ------
721       731        741        751        761
```

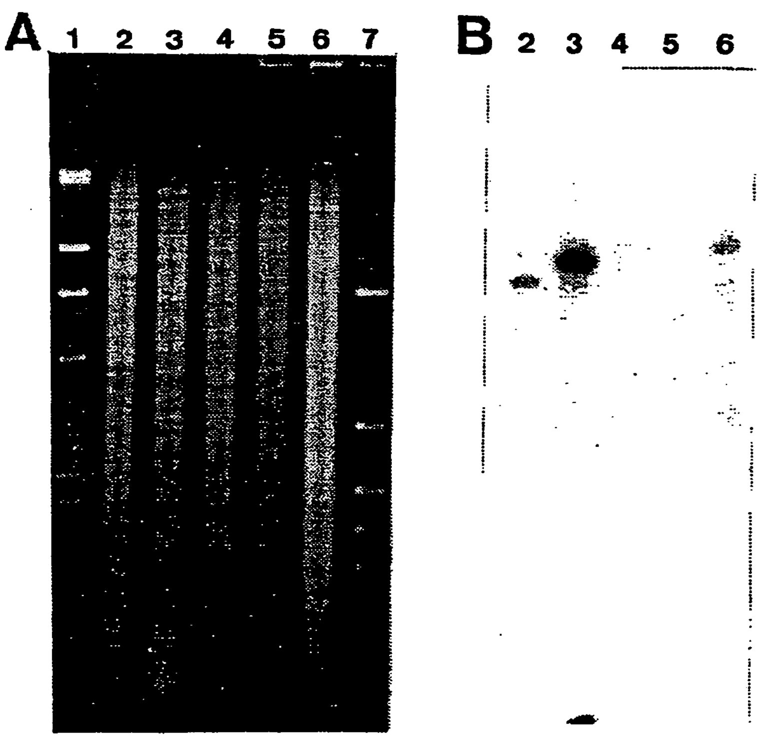

FIG. 7

FIGURE 8(1)

```
              1                                                                          70
(MTKATG)      MPEQHPPITE TTTGAASNGC PVVGHMKYPV EGGGNQDWWP NRLNLKVLHQ NPAVADPMGA AFDYAAEVAT
(ECKATG)      ..MSTSDDIH NTTATGKCPF HQGGHDQSAG AGTTTRDWWP NQLRVDLLNQ HSNRSNPLGE DFDYRKEF..
(STKATG)      ..MSTTDDTH NTLSTGKCPF HQGGHDRSAG AGTASRDWWP NQLRVDLLNQ HSNRSNPLGE DFDYRKEF..
(BSPERA)      ......MENQ NRQNAAQCPF HESVTNQSS. NRTTNKDWWP NQLNLSILHQ HDRKTNPHDE EFNYAEEFQ.
  (CCP)       .......... .......... .......... .......... ...TTPLVHV ASVEKGRSYE DFQ.......
CONSENSUS     --MST-DDTH NTT---KCPF HQGGHDQSAG AGTTNRDWWP NQL--DLLHQ HSNRSNPLGE DFDY-KEF--

              71                                                                        140
(MTKATG)      SRLD...ALT RDIEEVMTTS QPWWPADYGH YGPLFIRMAW HAAGTYRIHD GRGGAGGGMQ RFAPLNSWPD
(ECKATG)      SKLDYY.GLK KDLKALLTES QPWWPADWGS YAGLFIRMAW HGAGTYRSID GRGGAGRGQQ RFAPLNSWPD
(STKATG)      SKLDYYSALK GDLKALLTDS QPWWPADWGS YVGLFIRMAW HGAGTYRSID GRGGAGRGQQ RFAPLNSWPD
(BSPERA)      .KLDYW.ALK EDLRKLMTES QDWWPADYGH YGPLFIRMAW HSAGTYRIGD GRGGASTGTQ RFAPLNSWPD
  (CCP)       .KVYNAIALK ......LRED DEY..DNYIG YGPVLVRLAW HISGTWDKHD NTGGSYGGTY RFKKEFNDPS
CONSENSUS     SKLDYY-ALK -DLKALLTES QPWWPADYG- YGPLFIRMAW HGAGTYR--D GRGGAG-G-Q RFAPLNSWPD
                                                    R    W H(108)

              141                                                                       210
(MTKATG)      NASLDKARRL LWPVKKKYGK KLSWADLIVF AGNRCARNRW ASRRSGSASG ...VDQWETD .EVYWGKEAT
(ECKATG)      NVSLDKARRL LWPIKQKYGQ KISWADLFIL AGNVALENSG FRTFGFGAGR ...EDVWEPD LDVNWGDEKA
(STKATG)      TVSLDKARRL LWPIKQKYGQ KISWADLFIL AGNVALENSG FRTFGFGAGR ...EDVWEPD LDVNWGDEKA
(BSPERA)      NANLDKARRC YGRSKRNTGT K.SLGPICSF WRAMSLLNRW VEKRLDSAAG PLTSGIRKKT FIGDRKKSGS
  (CCP)       NAGLQNGFKF LEPIHKEFP. WISSGDLFSL GGVTAVQEMQ GPKIPWRCGR VDTPEDTTPD ........NG
CONSENSUS     NASLDKARRL LWPIK-KYGQ KISWADLFIL AGNVALEN-- FR--GF-AGR --TEDVWEPD LDVNWG-EKA
              N(138)

              211                                                                       280
(MTKATG)      WLGDDGYSVS DLENPLAAVQ MGLIYVNPEA PNGNPDPMAA AVDIRETFRR MAMNDVETAA LIVGGHTFGK
(ECKATG)      WLTHR.HPEA LAKAPLGATE MGLIYVNPEG PDHSGEPLSA AAAIRATFGN MGMNDEETVA LIAGGHTLGK
(STKATG)      WLTHR.HPEA LAKAPLGATE MDLIYVTPEG PNHSGEPLSA AAAIRATFGN MGMNDEETVA LIAGGHTLGK
(BSPERA)      PLNAIPVIAS SKTRSPRANG VNLRQPRRAG RQAGSKSRGI SA...ETFRR MGMNDEETVA LIAGGHTFGK
  (CCP)       RL........ .......... .......... .....PDADKD AGYVRTFFQR LNMNDREVVA LM.GAHALGK
CONSENSUS     WLTHR-HPE- LAKAPLGATE MGLIYVNPEG PNHS--PLSA AAAIR-TF-R MGMNDEETVA LIAGGHTLGK
                                                                                    H(269)

              281                                                                       350
(MTKATG)      THGAGPADLV GPEPEAAPLE QMGLGWKSSY GTGTGKDAIT SGIEVVWTNT PTKWDNSFLE ILYGYEWELT
(ECKATG)      THGAGPTSNV GPDPEAAPIE EQGLGWASTY GSGVGADAIT SGLEVVWTQT PTQWSNYFFE NLFKYEWVQT
(STKATG)      THGPAAASHV GADPEAAPIE AQGLGWASSY GSGVGADAIT SGLEVVWTQT PTQWSNYFFE NLFKYEWVQT
(BSPERA)      AHRGGPATHV GPEPEAAPIE AQGLGWISSY GKGKGSDTIT SGIEGAWTPT PTQWDTSYFD MLFGYDWWLT
  (CCP)       TH........ .......... .......... .......LKN SGYEGPWGAA NNVFTNEFYL NLLNEDWKLE
CONSENSUS     THGAGPASHV GP-PEAAPIE AQGLGWASSY GSGVGADAIT SG-EVVWTQT PTQW-N-FFE NLF-YEWVLT
              TH(275)                                            W(320)

              351                                                                       420
(MTKATG)      KSPAGAWQYT AKDGAGAGTI PDPFGGPGR. ..SPTMLATD LSLRVDPIYE RITRRWLEHP EELADEFRKA
(ECKATG)      RSPAGAIQFE AVD..APEII PDPFDPSKKR ..KPTMLVTD LTLRFDPEFE KISRRFLNDP QAFNEAFARA
(STKATG)      RSPAGAIQFE AVD..APDII PDPFDPSKKR XXKPTMLVTD LTLRFDPEFE KISRRFLNDP QAFNEAFARA
(BSPERA)      KSPAGAWQWM AVDPDEKDLA PDAEDPSKK. .VPTMMMTTD LALRFDPEYE KIARRFHQNP EEFAEAFARA
  (CCP)       KNDANNEQWD SKSGY..... .......... ....MMLPTD YSLIQDPKYL SIVKEYANDQ DKFFKDFSKA
CONSENSUS     KSPAGA-Q-E AVDG-APDII PDPFDPSKKR --KPTMLVTD L-LRFDPEYE KISRRFLNDP E-F-EAFARA
                                                         D(380)
```

FIGURE 8(2)

```
           421                                                                    490
{MTKATG}   WYKLIHRDM. ...GPVARYL GPLVPKQTLL WQDPVPAVST TSSAKQIASL KSQIRASGLT VSQLVSTAWA
{ECKATG}   WFKLTHRDM. ...GPKSRYI GPEVPKEDLI WQDPLPQPIY NPTEQDIIDL KFAIADSGLS VSELVSVAWA
{STKATG}   WFKLTHRDM. ...GPKARYI GPEVPKEDLI WQDPLPQPLY QPTQEDIINL KAAIAASGLS ISEMVSVAWA
{BSPERA}   WFKLTHRDM. ...GPKTRYL GPEVPKEDFI WQDPIPEVDY ELTEAEIEEI KAKILNSGLT VSELVKTAWA
  {CCP}    FEKLLENGIT FPKDAPSPFI FKTLEEQGL. .......... .......... .......... ..........
CONSENSUS  WFKLTHRDM- ---GPK-RYI GPEVPKEDLI WQDP-PQ--Y -PTE-DII-L KAAIAASGL- VSELVS-AWA

           491                                                                    560
{MTKATG}   AASSFRGSDK RGGA.NGGRI RLQPQVGWEV NDPDGSAQGH SHPEEIQESF TRRGNIKVSF ADLVVLGGCA
{ECKATG}   SASTFRGGDK RGGA.NGARL ALMPQRDWDV N..AAAVRAL PVLEKIQ... ..KESGKASL ADIIVLAGVV
{STKATG}   SASTFRGGDK RGGA.NGARL ALAPQRDWDV N..AVAARVL PVLEEIQ... ..KTTNKASL ADIIVLAGVV
{BSPERA}   SAA..RSATR ISAATNGRRI RLAPQKDWEV NEPERLAKVL SVLRGHPA.. ..RTAEKSKH RRLDRLGGTL
  {CCP}    .......... .......... .......... .......... .......... .......... ..........
CONSENSUS  SASTFRGGDK RGGA-NGAR- -LAPQRDW-V N-P--AARVL -VLEEIQ--- ---T--KASL AD-IVL-GVV

           561                                                                    630
{MTKATG}   PLEKAAKAAG HNITVPF... TPGPHDASQE QTDVESFAVL EPKADGFRN. ...YLGKGNR CRPSTSLLDK
{ECKATG}   GVEKAASAAG LSIHVPF... APGRVDARQD QTDIEMFELL EPIADGFRN. ...YRARLDV STTESLLIDK
{STKATG}   GIEQAAAAAR VSIHVPF... PPGRVDARHD QTDIEMFSLL EPIADGFRN. ...YRARLDV STTESLLIDK
{BSPERA}   RWKRQPATPA LMSKCHFSLA AAMRHKSKPM SKALPCWNRS QMASATIKSK STRFRRKSCS STKPSSSADR
  {CCP}    .......... .......... .......... .......... .......... .......... ..........
CONSENSUS  G-EKAAAAAG LSIHVPF--- APGR-DARQD QTDIEMF-LL EPIADGFRN- ---YRA-LDV STTES-LIDK

           631                                                                    700
{MTKATG}   ANLLTLSAPE MTVLVGGLRV LGANYKRLPL GVFTEASESL TNDFFVNLLD MGITWEPSPA DDGTYQGKD.
{ECKATG}   AQQLTLTAPE MTALVGGMRV LGGNFDGSKN GVFTDRVGVL SNDFFVNLLD MRYEWKATDE SKELFEGRDR
{STKATG}   AQQLTLTAPE MTVLVGGMRV LGTNFDGSQN GVFTDKPGVL STDFFANLLD MRYEWKPTDD ANELFEGRDR
{BSPERA}   PRNDGLSWR. .......FAR VGPNYRHLPH GVFTDRIGVL TNDFFVNLLD MNYEWVPTDS ..GIYEIRDR
  {CCP}    .......... .......... .......... .......... .......... .......... ..........
CONSENSUS  AQQLTL-APE MTVLVGGMRV LG-N-DG-PN GVFTDR-GVL -NDFFVNLLD MRYEWKPTD- ---L-EGRDR

           701                                                                 767
{MTKATG}   GSGKVKWTGS RVDLVFGSNS ELRALVEVYA PMTRQAKFVT GFVAAWDKVM NLDRFDVR.. .......
{ECKATG}   ETGEVKFTAS RADLVFGSNS VLRAVAEVYA SSDAHEKFVK DFVAAWVKVM NLDRFDLL.. .......
{STKATG}   LTGEVKYTAT RADLVFGSNS VLRALAEVYA CSDAHEKFVK DFVAAWVKVM NLDRFDLQ.. .......
{BSPERA}   KTGEVRWTAT RVDLIFGSNS ILRSYAEFYA QDDNQEKFVR DFINAWVKVM NADRFDLVKK ARESVTA
  {CCP}    .......... .......... .......... .......... .......... .......... .......
CONSENSUS  -TGEVKWTA- R-DLVFGSNS VLRALAEVYA -SDA-EKFVK DFVAAWVKVM NLDRFDL--- -------
```

SUBSTITUTE SHEET

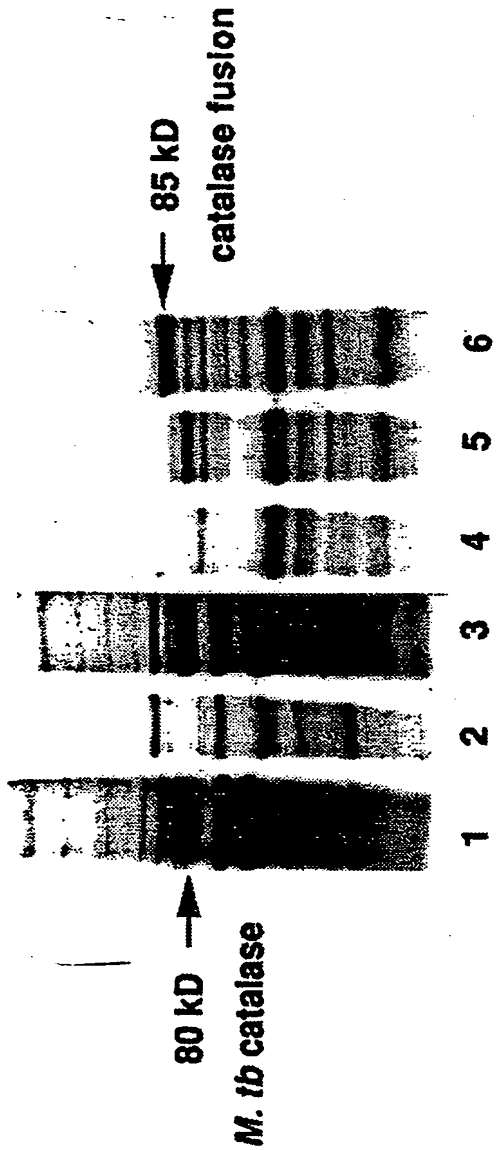

FIG. 9

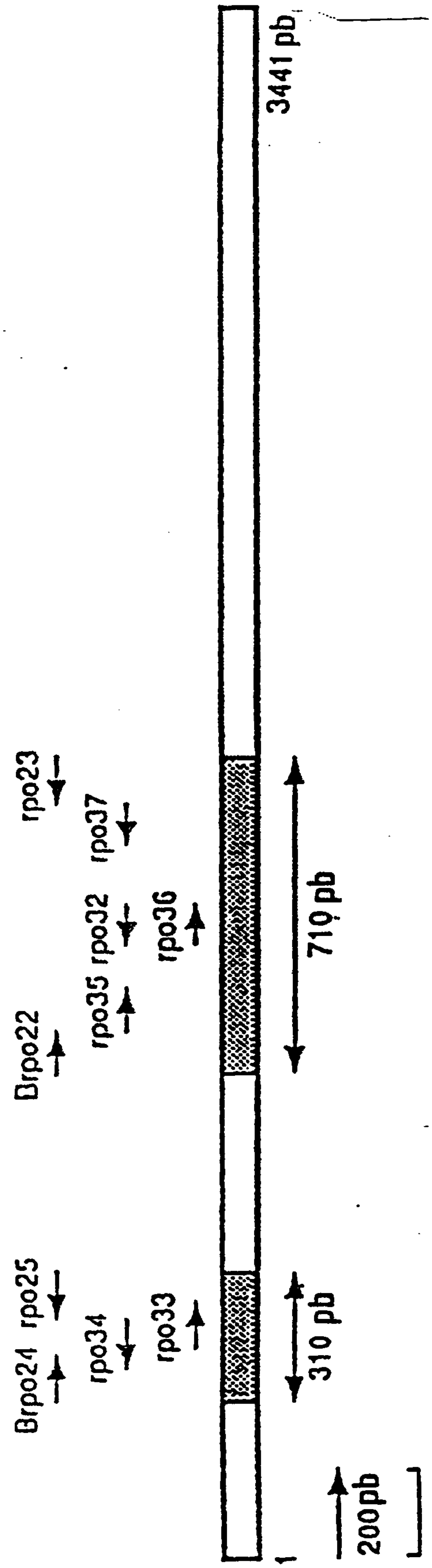
3441 pb
rpo23
rpo37
rpo32
rpo36
rpo35
Brpo22
710 pb
rpo25
rpo34
rpo33
Brpo24
310 pb
200pb

FIG. 10

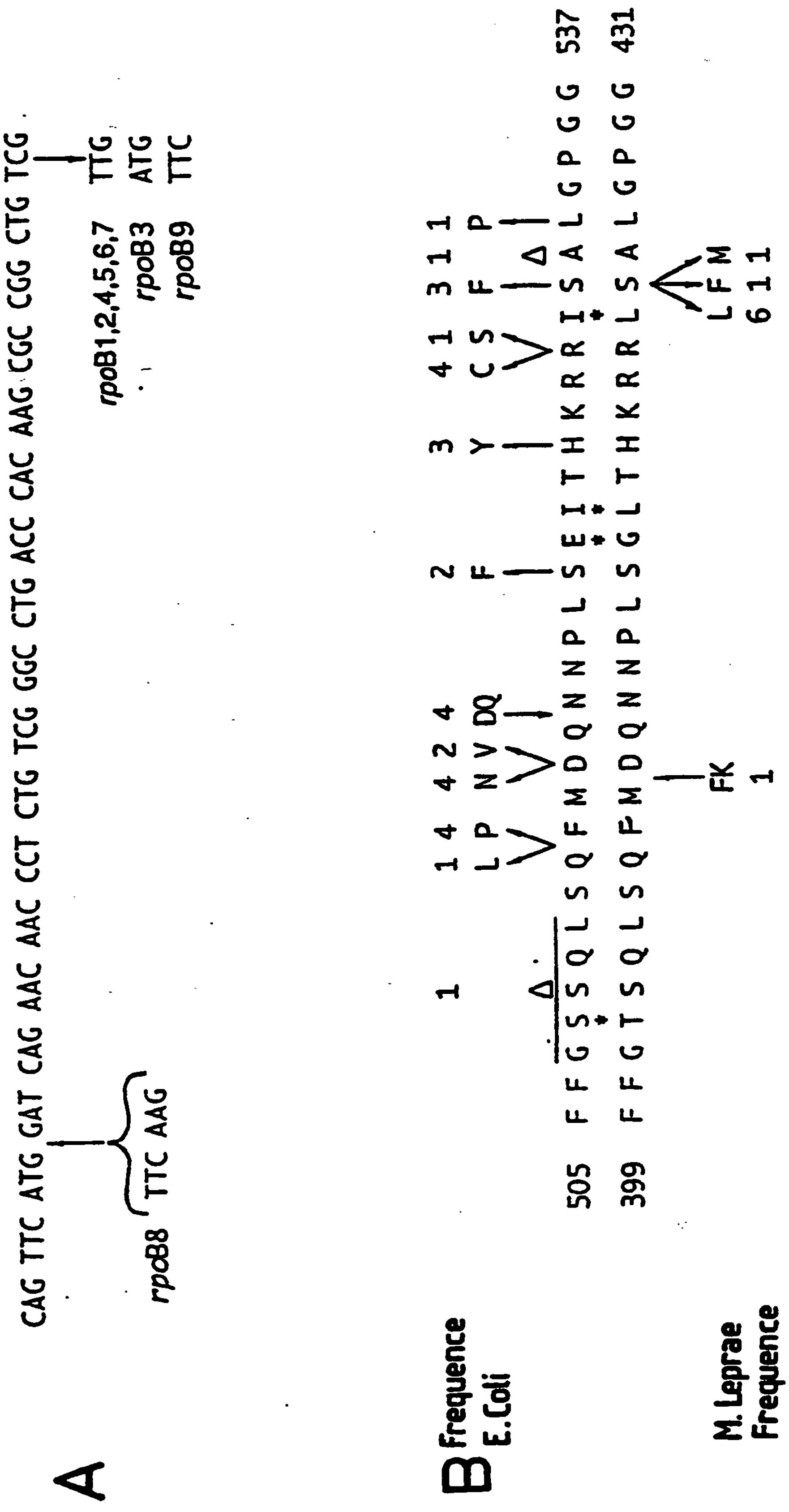
A
CAG TTC ATG GAT CAG AAC AAC CCT CTG TCG GGC CTG ACC CAC AAG CGC CGG CTG TCG
rpoB8 TTC AAG
rpoB1,2,4,5,6,7 TTG
rpoB3 ATG
rpoB9 TTC
B
Frequence
E.Coli
1 1 4 4 2 4 2 3 4 1 3 1 1
L P N V DQ F Y C S F P
Δ Δ
505 F F G S S Q L S Q F M D Q N N P L S E I T H K R R I S A L G P G G 537
399 F F G T S Q L S Q F M D Q N N P L S G L T H K R R L S A L G P G G 431
FK LFM
M. Leprae
Frequence
1 6 1 1

FIG. 11

MetProGlyAlaProAsnArgIleSerPheAlaLysLeuArgGluProLeuGluValPro
GTGCCCGGCGCGCCCAACCGAATTTCATTTGCCAAGCTCCGCGAACCGCTTGAGGTTCCG 60

GlyLeuLeuAspValGlnThrAspSerPheGluTrpLeuIleGlySerProCysTrpArg
GGGCTACTTGATGTGCAGACTGATTCATTTGAGTGGTTGATCGGATCGCCGTGCTGGCGT 120

AlaAlaAlaAlaSerArgGlyAspLeuLysProValGlyGlyLeuGluGluValLeuTyr
GCAGCGGCCGCAAGCCGCGGCGATCTCAAGCCGGTGGGTGGTCTCGAAGAGGTGCTCTAC 180

GluLeuSerProIleGluAspPheSerGlySerMetSerLeuSerPheSerAspProArg
GAGCTGTCGCCGATCGAGGATTTCTCCGGCTCAATGTCATTGTCTTTCTCCGATCCCCGT 240

PheAspGluValLysAlaProValGluGluCysLysAspLysAspMetThrTyrAlaAla
TTTGACGAAGTCAAGGCGCCCGTCGAAGAGTGCAAAGACAAGGACATGACGTACGCGGCC 300

ProLeuPheValThrAlaGluPheIleAsnAsnAsnThrGlyGluIleLysSerGlnThr
CCGCTGTTCGTCACGGCCGAGTTCATCAACAACAACACCGGGGAGATCAAGAGCCAGACG 360

ValPheMetGlyAspPheProMetMetThrGluLysGlyThrPheIleIleAsnGlyThr
GTGTTTATGGGCGACTTCCCTATGATGACTGAGAAGGGAACCTTCATCATCAACGGGACC 420

GluArgValValValSerGlnLeuValArgSerProGlyValTyrPheAspGluThrIle
GAGCGTGTCGTCGTTAGCCAGCTGGTGCGCTCCCCTGGAGTATACTTCGACGAGACGATC 480

AspLysSerThrGluLysThrLeuHisSerValLysValIleProSerArgGlyAlaTrp
GACAAGTCCACAGAAAAGACGCTGCATAGTGTCAAGGTGATTCCCAGCCGCGGTGCCTGG 540

LeuGluPheAspValAspLysArgAspThrValGlyValArgIleAspArgLysArgArg
TTGGAATTCGATGTCGATAAACGCGACACCGTCGGTGTCCGCATTGACCGGAAGCGCCGG 600

GlnProValThrValLeuLeuLysAlaLeuGlyTrpThrSerGluGlnIleThrGluArg
CAACCCGTCACGGTGCTTCTCAAAGCGCTAGGTTGGACCAGTGAGCAGATCACCGAGCGT 660

PheGlyPheSerGluIleMetArgSerThrLeuGluLysAspAsnThrValGlyThrAsp
TTCGGTTTCTCCGAGATCATGCGCTCGACGCTGGAGAAGGACAACACAGTTGGCACCGAC 720

GluAlaLeuLeuAspIleTyrArgLysLeuArgProGlyGluProProThrLysGluSer
GAGGCGCTGCTAGACATCTATCGTAAGTTGCGCCCAGGTGAGCCGCCGACTAAGGAGTCC 780

AlaGlnThrLeuLeuGluAsnLeuPhePheLysGluLysArgTyrAspLeuAlaArgVal
GCGCAGACGCTGTTGGAGAACCTGTTCTTCAAGGAGAAACGCTACGACCTGGCCAGGGTT 840

GlyArgTyrLysValAsnLysLysLeuGlyLeuHisAlaGlyGluLeuIleThrSerSer
GGTCGTTACAAGGTCAACAAGAAGCTCGGGTTGCACGCCGGTGAGTTGATCACGTCGTCC 900

ThrLeuThrGluGluAspValValAlaThrIleGluTyrLeuValArgLeuHisGluGly
ACGCTGACCGAAGAGGATGTCGTCGCCACCATAGAGTACCTGGTTCGTCTGCATGAGGGT 960

GlnSerThrMetThrValProGlyGlyValGluValProValGluThrAspAspIleAsp
CAGTCGACAATGACTGTCCCAGGTGGGGTAGAAGTGCCAGTGGAAACTGACGATATCGAC 1020

HisPheGlyAsnArgArgLeuArgThrValGlyGluLeuIleGlnAsnGlnIleArgVal
CACTTCGGCAACCGCCGGCTGCGCACGGTCGGCGAATTGATCCAGAACCAGATCCGGGTC 1080

GlyMetSerArgMetGluArgValValArgGluArgMetThrThrGlnAspValGluAla
GGTATGTCGCGGATGGAGCGGGTGGTCCGGGAGCGGATGACCACCCAGGACGTCGAGGCG 1140

FIG. ~~3~~ 12(1)

IleThrProGlnThrLeuIleAsnIleArgProValValAlaAlaIleLysGluPhePhe
ATCACGCCGCAGACGCTGATCAATATCCGTCCGGTGGTCGCCGCTATCAAGGAATTCTTC 1200

GlyThrSerGlnLeuSerGlnPheMetAspGlnAsnAsnProLeuSerGlyLeuThrHis
GGCACCAGCCAGCTGTCGCAGTTCATGGATCAGAACAACCCTCTGTCGGGCCTGACCCAC 1260

LysArgArgLeuSerAlaLeuGlyProGlyGlyLeuSerArgGluArgAlaGlyLeuGlu
AAGCGCCGGCTGTCGGCGCTGGGCCCGGGTGGTTTGTCGCGTGAGCGTGCCGGGCTAGAG 1320

ValArgAspValHisProSerHisTyrGlyArgMetCysProIleGluThrProGluGly
GTCCGTGACGTGCACCCTTCGCACTACGGCCGGATGTGCCCGATCGAGACTCCGGAGGGC 1380

ProAsnIleGlyLeuIleGlySerLeuSerValTyrAlaArgValAsnProPheGlyPhe
CCGAACATAGGTCTGATCGGTTCATTGTCGGTGTACGCGCGGGTCAACCCCTTCGGGTTC 1440

IleGluThrProTyrArgLysValValAspGlyValValSerAspGluIleGluTyrLeu
ATCGAAACACCGTACCGCAAAGTGGTTGACGGTGTGGTCAGCGACGAGATCGAATACTTG 1500

ThrAlaAspGluGluAspArgHisValValAlaGlnAlaAsnSerProIleAspGluAla
ACCGCTGACGAGGAAGACCGCCATGTCGTGGCGCAGGCCAACTCGCCGATCGACGAGGCC 1560

GlyArgSerSerSerArgAlaCysTrpValArgArgLysAlaGlyGluValGluTyrVal
GGCCGTTCCTCGAGCCGCGCGTGTTGGGTGCGCCGCAAGGCGGGCGAGGTGGAGTACGTG 1620

AlaSerSerGluValAspTyrMetAspValSerProArgGlnMetValSerValAlaThr
GCCTCGTCCGAGGTGGATTACATGGATGTCTCGCCACGCCAGATGGTGTCGGTGGCCACA 1680

AlaMetIleProPheLeuGluHisAspAspAlaAsnArgAlaLeuMetGlyAlaAsnMet
GCGATGATTCCGTTCCTTGAGCACGACGACGCCAACCGTGCCCTGATGgGcgCTAACATG 1740

GlnArgGlnAlaValProLeuValArgSerGluArgProLeuValGlyThrGlyMetGlu
CAGcgCCAAGCGGTTCCGTTGGTGCGCAGCGAACGACCGTTGGTGGGTACCGGTATGGAG 1800

LeuArgAlaAlaIleAspAlaGlyHisValValValAlaGluLysSerGlyValIleGlu
TTGCGCGCGGCCATCGACGCTGGCCACGTCGTCGTTGCGGAGAAGTCCGGGGTGATCGAG 1860

GluValSerAlaAspTyrIleThrValMetAlaAspAspGlyThrArgArgThrTyrArg
GAGGTTTCCGCCGACTACATCACCGTGATGGCCGATGACGGCACCCGGCGGACTTATCGG 1920

MetArgLysPheAlaArgSerAsnHisGlyThrCysAlaAsnGlnSerProIleValAsp
ATGCGTAAGTTCGCGCGCTCCAACCACGGCACCTGCGCCAACCAGTCCCCGATCGTGGAT 1980

AlaGlyAspArgValGluAlaGlyGlnValIleAlaAspGlyProCysThrGluAsnGly
GCGGGGGATCGGGTCGAGGCCGGCCAAGTGATTGCTGACGGTCCGTGCACTGAGAACGGC 2040

GluMetAlaLeuGlyLysAsnLeuLeuValAlaIleAsnAlaValGlyGlySerThrThr
GAGATGGCGTTGGGCAAGAACTTGCTGGTGGCGATCAATGCCGTGGGAGGGTCAACAACT 2100

AsnGluAspAlaIleIleLeuSerAsnArgLeuValGluGluAspValLeuThrSerIle
AACGAGGATGCGATCATCCTGTCTAACCGACTGGTCGAAGAGGACGTGCTTACTTCGATT 2160

HisIleGluGluHisGluIleAspAlaArgAspThrLysLeuGlyAlaGluGluIleThr
CACATTGAGGAGCATGAGATCGACGCCCGTGACACCAAGCTGGGTGCTGAGGAGATCACC 2220

ArgAspIleProAsnValSerAspGluValLeuAlaAspLeuAspGluArgGlyIleVal
CGGGACATTCCCAACGTCTCCGATGAGGTGCTAGCCGACTTGGACGAGCGGGGCATCGTG 2280

ArgIleGlyAlaGluValArgAspGlyAspIleLeuValGlyLysValThrProLysGly
CGGATTGGCGCGGAGGTTCGTGACGGTGATATCCTGGTTGGCAAGGTCACCCCGAAGGGG 2340

FIG. ~~3~~ ~~suite~~ 12(2)

```
GluThrGluLeuThrProGluGluArgLeuLeuArgAlaIlePheGlyGluLysAlaArg
GAAACTGAGCTGACACCGGAAGAGCGGTTGCTGCGGGCGATCTTCGGCGAAAAGGCCCGC    2400

GluValArgAspThrSerLeuLysValProHisGlyGluSerGlyLysValIleGlyIle
GAGGTCCGTGACACGTCGCTGAAGGTGCCACACGGCGAATCCGGCAAGGTGATCGGCATT    2460

ArgValPheSerHisGluAspAspAspGluLeuProAlaGlyValAsnGluLeuValArg
CGGGTGTTCTCCCATGAGGATGACGACGAGCTGCCCGCCGGCGTCAACGAGCTGGTCCGT    2520

ValTyrValAlaGlnLysArgLysIleSerAspGlyAspLysLeuAlaGlyArgHisGly
GTCTACGTAGCCCAGAAGCGCAAGATCTCTGACGGTGACAAGCTGGCTGGGCGGCACGGC    2580

AsnLysGlyValIleGlyLysIleLeuProAlaGluAspMetProPheLeuProAspGly
AACAAGGGCGTGATCGGCAAGATCCTGCCTGCCGAGGATATGCCGTTTCTGCCAGACGGC    2640

ThrProValAspIleIleLeuAsnThrHisGlyValProArgArgMetAsnValGlyGln
ACCCCGGTGGACATCATCCTCAACACTCACGGGGTGCCGCGGCGGATGAACGTCGGTCAG    2700

IleLeuGluThrHisLeuGlyTrpValAlaLysSerGlyTrpLysIleAspValAlaGly
ATCTTGGAAACCCACCTTGGGTGGGTAGCCAAGTCCGGCTGGAAGATCGACGTGGCCGGC    2760

GlyIleProAspTrpAlaValAsnLeuProGluGluLeuLeuHisAlaAlaProAsnGln
GGTATACCGGATTGGGCGGTCAACTTGCCTGAGGAGTTGTTGCACGCTGCGCCCAACCAG    2820

IleValSerThrProValPheAspGlyAlaLysGluGluGluLeuGlnGlyLeuLeuSer
ATCGTGTCGACCCCGGTGTTCGACGGCGCCAAGGAAGAGGAACTACAGGGCCTGTTGTCC    2880

SerThrLeuProAsnArgAspGlyAspValMetValGlyGlyAspGlyLysAlaValLeu
TCCACGTTGCCCAACCGCGACGGCGATGTGATGGTGGGCGGCGACGGCAAGGCGGTGCTC    2940

PheAspGlyArgSerGlyGluProPheProTyrProValThrValGlyTyrMetTyrIle
TTCGATGGGCGCAGCGGTGAGCCGTTCCCTTATCCGGTGACGGTTGGCTACATGTACATC    3000

MetLysLeuHisHisLeuValAspAspLysIleHisAlaArgSerThrGlyProTyrSer
ATGAAGCTGCACCACTTGGTGGACGACAAGATCCACGCCCGCTCCACCGGCCCGTACTCG    3060

MetIleThrGlnGlnProLeuGlyGlyLysAlaGlnPheGlyGlyGlnArgPheGlyGlu
ATGATTACCCAGCAGCCGTTGGGTGGTAAGGCACAGTTCGGTGGCCAGCGATTCGGTGAG    3120

MetGluCysTrpAlaMetGlnAlaTyrGlyAlaAlaTyrThrLeuGlnGluLeuLeuThr
ATGGAGTGCTGGGCCATGCAGGCCTACGGTGCGGCCTACACGCTGCAGGAGCTGTTGACC    3180

IleLysSerAspAspThrValGlyArgValLysValTyrGluAlaIleValLysGlyGlu
ATCAAGTCCGACGACACCGTCGGTCGGGTCAAGGTTTACGAGGCTATCGTTAAGGGTGAG    3240

AsnIleProGluProGlyIleProGluSerPheLysValLeuLeuLysGluLeuGlnSer
AACATCCCCGAGCCGGGCATCCCCGAGTCGTTCAAGGTGCTGCTCAAGGAGTTACAGTCG    3300

LeuCysLeuAsnValGluValLeuSerSerAspGlyAlaAlaIleGluLeuArgGluGly
CTGTGTCTCAACGTCGAGGTGCTGTCGTCCGACGGTGCGGCGATCGAGTTGCGCGAAGGT    3360

GluAspGluAspLeuGluArgAlaAlaAlaAsnLeuGlyIleAsnLeuSerArgAsnGlu
GAGGATGAGGACCTCGAGCGGGCTGCGGCCAACCTCGGTATCAACTTGTCCCGCAACGAA    3420

SerAlaSerIleGluAspLeuAla***
TCGGCGTCCATAGAAGATCTGGCTTAG    3447
```

FIG.~~3 suite~~ 12(3)

```
GlyAsnArgArgLeuArgThrValGlyGluLeuIleGlnAsnGlnIleArgValGlyMet
GGCAACCGCCGCCTGCGTACGGTCGGCGAGCTGATCCAAAACCAGATCCGGGTCGGCATG      60

SerArgMetGluArgValValArgGluArgMetThrThrGlnAspValGluAlaIleThr
TCGCGGATGGAGCGGGTGGTCCGGGAGCGGATGACCACCCAGGACGTGGAGGCGATCACA     120

ProGlnThrLeuIleAsnIleArgProValValAlaAlaIleLysGluPhePheGlyThr
CCGCAGACGTTGATCAACATCCGGCCGGTGGTCGCCGCGATCAAGGAGTTCTTCGGCACC     180

SerGlnLeuSerGlnPheMetAspGlnAsnAsnProLeuSerGlyLeuThrHisLysArg
AGCCAGCTGAGCCAATTCATGGACCAGAACAACCCGCTGTCGGGGTTGACGCACAAGCGC     240

ArgLeuSerAlaLeuGlyProGlyGlyLeuSerArgGluArgAlaGlyLeuGluValArg
CGACTGTCGGCGCTGGGGCCCGGCGGTCTGTCACGTGAGCGTGCCGGGCTGGAGGTCCGC     300

AspValHisProSerHisTyrGlyArgMetCysProIleGluThrProGluGlyProAsn
GACGTGCACCCGTCGCACTACGGCCGGATGTGCCCGATCGAAACCCCTGAGGGGCCCAAC     360

IleGlyLeuIleGlySerLeuSerValTyrAlaArgValAsnProPheGlyPheIleGlu
ATCGGTCTGATCGGCTCGCTGTCGGTGTACGCGCGGGTCAACCCGTTCGGGTTCATCGAA     420

ThrProTyrArg
ACGCCGTACCGC      432
```

FIGURE 13

FIGURE 14(1)

```
atgcccgatcacagggcactgcggcagggaataattgcactacgccaacatgttaacaac   20752

                            1
                            M  P  T  I  Q  Q  L  V  R  K  G
gaacacaatttacctgggagccggtatatgcccaccattcagcagctggtacgcaagggt   20812
                              ____________________c---------
                              ML51

 R  R  D  K  I  G  K  V  K  T  A  A  L  K  G  N  P  Q  R  R
cgtcgagacaagattggcaaggtcaagactgcggctctgaagggcaacccacagcgtcgc   20872
-----g--------ca-t-----------c----------------g---g--------t
                S                             S

 G  V  C  T  R  V  Y  T  S  T  P  K  K  P  N  S  A  L  R  K
ggtgtttgcacccgtgtgtacacttccaccccgaagaagccgaactcggcgcttcgcaag   20932
-----a--------c--------ca----t--------------------------g---
                         T

 V  A  R  V  K  L  T  S  Q  V  E  V  T  A  Y  I  P  G  E  G
gttgcccgcgtgaagctgacgagtcaggttgaggtcacagcgtacataccaggcgagggt   20992
---------------t-------------c--------g--------t--c-------cg
                                                          A

 H  N  L  Q  E  H  S  M  V  L  V  R  G  G  R  V  K  D  L  P
cacaacctacaggaacactccatggtgttggtgcgtggtggccgggtgaaagatctgcct   21052
--------g-----g-----g------c-------c--c-----------g--c------
```

```
G  V  R  Y  K  I  I  R  G  S  L  D  T  Q  G  V  K  N  R  K
ggtgtgcgttacaaaatcattcgcggttcgctcgacacccagggtgtcaagaaccggaag   21112
--------c-----g____________________________
                                           ML52

Q  A  R  S  R  Y  G  A  K  K  E  K  S  *
caggctcgtagccgctatggagccaagaaggagaagagctga   21154
```

FIGURE 14(2)

```
                                    10
R  K  G   R  R  D  K   I  G  K   V  K  T   A  A  L  K
CGCAAGGGTC GTCGAGACAA GATTGGCAAG GTCAAGACCG CGGCTCTGAA
   10          15                    20              25

 G   N P   Q  R  R   G   V  C   T R   V  Y   T  S T
GGGCAGCCCG CAGCGTCGTG GTGTATGCAC CCGCGTGTAC ACCACCACTC
                30          35              40
  42
P  K  K  P   N   S A   L  R  K   V  A  R   V  K L  T
CGAAGAAGCC GAACTCGGCG CTTCGGAAGG TTGCCCGCGT GAAGTTGACG
        45                50          55

 S  Q  V   E  V  T  A   Y  I  P   G  E  G   H N  L  Q
 AGTCAGGTCG AGGTCACGGC GTACATTCCC GGCGAGGCGC ACAACCTGCA
   60           65               70              75

 E  H  S   M  V  L   V  R  G   G  R  V  K  D  L  P
GGAGCACTCG ATGGTGCTGG TGCGCGGCGG CCGGGTGAAG GACCTGCCTG
                80              85              90

 G  V  R  Y   K
 GTGTGCGCTAC AAG.
        95
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FIG. 15

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 93/01063

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68; //(C12Q1/68,C12R1:32)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.-5	C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category°	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	JOURNAL OF MOLECULAR BIOLOGY vol. 202, no. 1, August 1988, LONDON pages 45 - 58 JIN ET AL. 'Mapping and sequencing of mutations in the Escherichia coli rpoB gene that lead to rifampicin resistance' see the whole document	1,16,17, 19
A		18,20
Y	WO,A,9 106 674 (SCOTGEN LTD) 16 May 1991 see page 3 - page 5	1,16,17, 20
A		3-8,18, 19
Y	EP,A,0 223 156 (HOECHST JAPAN LIMITED) 27 May 1987 see page 7 - page 10	1,16,17, 20
	-/--	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

3

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 AUGUST 1993	14.09.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	CEDER O.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	THE JOURNAL OF GENERAL MICROBIOLOGY vol. 60, no. 1, January 1970, LONDON pages 125 - 132 SRIPRAKASH ET AL. 'Isoniazid-resistant mutants of Mycobycterium tuberculosis H37RV: ...' cited in the application see abstract	2
P,X	NATURE vol. 358, no. 6387, 13 August 1992, LONDON pages 591 - 593 ZHANG ET AL. 'The catalase-peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis' cited in the application see the whole document	1-18
P,X	THE LANCET vol. 341, no. 8846, 13 March 1993, LONDON pages 647 - 650 TELENTI ET AL. 'Detection of rifampicin-resistance mutations in mycobacterium tuberculosis' see the whole document	1,16,17, 19
P,A	RESEARCH IN MICROBIOLOGY vol. 143, no. 7, September 1992, AMSTERDAM pages 721 - 730 HEYM ET AL. 'Isolation and characterization of isoniazid-resistant mutants of Mycobacterium smegmatis and M. aurum' cited in the application	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9301063
SA 74177

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9106674	16-05-91	None	
EP-A-0223156	27-05-87	AU-B- 587655	24-08-89
		AU-A- 6504286	14-05-87
		JP-A- 62201584	05-09-87

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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(54) Title: RAPID DETECTION OF ANTIBIOTIC RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS

(57) Abstract

Multidrug resistant strains of *Mycobacterium tuberculosis* represent a considerable threat to public health worldwide. Resistance to isoniazid (INH), rifampicin or analogues thereof, or streptomycin, i.e. key components of anti-tuberculosis regimens, need frequently to be detected. The invention involves the detection of a mutation in either the *katG* gene (isoniazid resistance), the *rpoB* gene (rifampicin resistance) or *rpsL* gene (streptomycin resistance).

* (Referred to in PCT Gazette No. 07/1994, Section II)

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RAPID DETECTION OF ANTIBIOTIC RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS

This invention relates to the rapid detection of strains of Mycobacterium tuberculosis that are resistant to antibiotics, particularly isoniazid, rifampicin and streptomycin. More particularly, this invention relates to a method of detecting antibiotic resistance in Mycobacterium tuberculosis, e.g. either as a result of mutations in the relevant genes or by nucleic acid hybridization. This invention also relates to a nucleic acid probe and a kit for carrying out the nucleic acid hybridization. The invention further relates to the chromosomal location of the katG gene and its nucleotide sequence.

BACKGROUND OF THE INVENTION

Despite more than a century of research since the discovery of Mycobacterium tuberculosis, the aetiological agent of tuberculosis, by Robert Koch, this disease remains one of the major causes of human morbidity and mortality. There are an estimated 3 million deaths annually attributable to tuberculosis (Snider, 1989), and although the majority of these are in developing countries, the disease is assuming renewed importance in the West due to the increasing number of homeless people and the impact of the AIDS epidemic (Chaisson et al., 1987; Snider and Roper, 1992).

Isonicotinic acid hydrazide or isoniazid (INH) has been used in the treatment of tuberculosis for the last forty years due to its exquisite potency against the members of the "tuberculosis" groups - Mycobacterium tuberculosis, M. bovis and M. africanum (Middlebrook, 1952; Youatt, 1969). Neither the precise target of the drug, nor its

mode of action, are known, and INH treatment results in the perturbation of several metabolic pathways. There is substantial evidence indicating that INH may act as an antimetabolite of NAD and pyridoxal phosphate (Bekierkunst and Bricker, 1967; Sriprakash and Ramakrishnan, 1970; Winder and Collins, 1968, 1969, 1970), and other data indicating that the drug blocks the synthesis of the mycolic acids, which are responsible for the acid-fast character of mycobacterial cell walls (Winder and Collins 1970; Quemard et al., 1991). Shortly after its introduction, INH-resistant isolates of *Mycobacterium tuberculosis* emerged and, on characterization, were often found to have lost catalase-peroxidase activity and to show reduced virulence in guinea pigs (Middlebrook et al., 1954; Kubica et al., 1968; Sriprakash and Ramakrishnan, 1970).

Very recently, INH-resistance has acquired new significance owing to a tuberculosis epidemic in the USA due to multidrug resistant (MDR) variants of *M. tuberculosis* (CDC, 1990; 1991a, b) and the demonstration that such strains were responsible for extensive nosocomial infections of HIV-infected individuals and health care workers (Snider and Roper, 1992). In view of the gravity of this problem, there exists a need in the art to determine the relationship between INH-resistance and catalase-peroxidase production.

More particularly, there is a need in the art to understand the molecular mechanisms involved in drug sensitivity. In addition, there is a need in the art to develop a simple test permitting the rapid identification of INH-resistant strains. Further, there is a need in the art for reagents to carry out such a test.

Rifampicin too is a majeur antibiotic used for the treatment of infections by mycobacterium, particularly Mycobacterium tuberculosis and Mycobacterium leprae. Because some mycobacteria grow slowly, possible rapid and efficient tests for the testing of resistance to rifampicin or analogues thereof must be made available. Likewise the invention aims at a rapid detection of strands of Mybobacterium tuberculosis which are resistant to streptomycin. Because of the development of resistance to streptomycin, the latter antibiotic has been used together with other antibiobics, e.g. isoniazid. Thus adequat treatment of tuberculosis should be preceded by rapid and efficient detection of resistances to the three majeur antibiotics, isoniazid, rifampicin and streptomycin.

SUMMARY OF THE INVENTION

Accordingly, this invention aids in fulfilling these needs in the art by providing a process for detecting in vitro the presence of cells of a Mycobacterium tuberculosis resistant to isoniazid and other drugs, such as rifampicin or analogues thereof, and streptomycin.

By analogues of rifampicin, a particularly meant derivatives of 3-formyl-rifamycin, particularly as a result of substitution the
rein for the sustituant present either in the naphtofuranonyl group or of the site chain at position 7 of the naphtofuranonyl group, or by the introduction or removal of a double band in the lateral chain.

In accordance with the invention, the detection of a resistance to isoniazid involves the detection of one or several litations in the katG gene of Mycobacterium

<u>tuberculosis</u>, particularly with respect to the nucleotide sequence of that same katG gene in mycobacterium tuberculosis that are not resistant to isoniazid.

Another process alternative for detecting <u>in vitro</u> the presence of nucleic acids of a <u>Mycobacterium tuberculosis</u> resistant to isoniazid, wherein the process comprises the steps of:

- contacting said nucleic acids previously made accessible to a probe if required under conditions permitting hybridization;
- detecting any probe that had hybridized to said nucleic acids;

wherein said probe comprises a nucleic acid sequence, which is 2.5 kb <u>Eco</u>RV-<u>Kpn</u>I fragment of plasmid pYZ56 or of part thereof, and wherein said fragment contains a <u>Bam</u>HI cleavage site, wherein said part is nonetheless sufficiently long to provide for the selectivity of the <u>in vitro</u> detection of a Mycobacterium tuberculosis resistant to isoniazid.

For instance, this process alternative comprises the steps of :

(A) depositing and fixing nucleic acids of the cells on a solid support, so as to make the nucleic acids accessible to a probe;

(B) contacting the fixed nucleic acids from step (A) with a probe under conditions permitting hybridization;

(C) washing the filter resulting from step (B), so as to eliminate any non-hybridized probe; and then

(D) detecting any hybridized probe on the washed filter resulting from step (C).

The probe comprises a nucleic acid sequence which is

present in a 2.5 kb EcoRV-KpnI fragment of plasmid pYZ56, wherein said fragment contains a BamHI cleavage site. This fragment has been found to be associated with intracellular DNA of isoniazid-sensitive Mycobacterium tuberculosis and is capable of distinguishing such antibiotic sensitive microorganisms from isoniazid-resistant Mycobacterium tuberculosis, which do not contain DNA that hybridizes with this fragment under the conditions described hereinafter.

This invention further provides nucleotide sequences, such as RNA and DNA, of isoniazid-resistant Mycobacterium tuberculosis encoding the region of the katG gene of Mycobacterium tuberculosis that imparts isoniazid sensitivity absent from isoniazid-resistant cells.

This invention also provides a probe consisting of a label, such as a radionuclide, bonded to a nucleotide sequence of the invention.

In addition, this invention provides a hybrid duplex molecule consisting essentially of a nucleotide sequence of the invention hydrogen bonded to a nucleotide sequence of complementary base sequence, such as DNA or RNA.

Also, this invention provides a process for selecting a nucleotide sequence coding for a catalase-peroxidase gene of Mycobacterium tuberculosis, or for a portion of such a nucleotide sequence, from a group of nucleotide sequences, which comprises the step of determining which of the nucleotide sequences hybridizes to a nucleotide sequence of the invention. The nucleotide sequence can be a DNA sequence or an RNA sequence. The process can include the step of detecting a label on the nucleotide sequence.

Further, this invention provides a kit for the

detection of Mycobacterium tuberculosis resistant to isoniazid. The kit comprises a container means containing a probe comprising a nucleic acid sequence, which is a 2.5 kb EcoRV-KpnI fragment of plasmid pYZ56, wherein the fragment contains a BamHI cleavage site. The kit also includes a container means containing a control preparation of nucleic acid.

The invention also covers compounds obtained as products of the action of the enzyme catalase, or a similar enzyme on isoniazid. The katG gene or a derivative of this gene which retains a similar activity can be used as a source of catalase protein. The new compounds are selected by reactivity on INH-resistant-mycobacterial strains by the antibiogram method such as described in H. David et al.'s "Methodes de laboratoire pour Mycobacteriologie clinique" edited by Pasteur Institute, ISBN N° 0995-2454.

BRIEF DESCRIPTION OF THE DRAWINGS

This invention will be described in greater detail by reference to the drawings in which:

Fig 1. shows the INH-resistant M. smegmatis strain, BH1 (Gayathri et al., 1975) (a derivative of strain MC^2-155) was transformed with a pool of M. tuberculosis-H37Rv shuttle cosmids (kindly provided by Dr. W.R. Jacobs, New York) and individual clones were scored for INH-susceptibility. Cosmid pBH4 consistently conferred drug susceptibility and the transformant overproduced catalase (assayed as in Heym, 1992). The restriction map of the DNA insert from pBH4 is shown along with that of the insert from pYZ55 - a plasmid containing katG of M. tuberculosis H37Rv, isolated on the basis of hybridization with an oligonucleotide probe

(5'-TTCATCCGCATGGCCTGGCACGGCGCGGGCACCTACCGC-3') designed to match the amino acid sequence from a conserved region of E. coli hydroperoxidase I (HPI). Restriction sites for the following enzymes are indicated : B, BamH1; C, Cla1; E, EcoRV; H, HindIII, K, Kpn1; M, Sma1; N, Not1; R, EcoR1; S, Sac1. Transformation of BH1 with a mycobacterial shuttle plasmid, pBAK14, Zhang et al., 1991, containing the 4.5 kb insert from pYZ55 similarly conferred INH-susceptibility. MIC's are also shown for BH1 transformed with subfragments derived from pYZ55 and inserted into pBAK14 in one (+) or other (-) orientation. The katG gene and the ability to confer INH-susceptibility both mapped to a 2.9 kb EcoRV-Kpn1 fragment (pBAK-KE+).

Fig. 2 shows extracts from M. tuberculosis H37Rv and from E. coli strains transformed with a variety of plasmid constructs that were prepared for activity gel analysis as described previously (Zhang et al., 1991). Non-denaturing gels containing 8% polyacrylamide were stained for catalase (panel A) and peroxidase (panel B) activities as described by Wayne and Diaz (Wayne et al., 1986). Lane 1, M. tuberculosis H37Rv; 2, E. coli UM2 (katE, katG; 3, E. coli UM2/pYZ55; 4, E. coli UM2/pYZ56 (the 2.9 kb EcoRV-Kpn1 fragment in pUC19, corresponding to pBAK-KE+ in Fig. 1); 5, E. coli UM2/pYZ57 (pYZ55 with a BamH1-Kpn1 deletion, corresponding to pBAK-KB+ in Fig. 1). M. tuberculosis catalase and peroxidase activities migrated as two bands under these conditions (lane 1); the same pattern was seen for the recombinant enzyme expressed by pYZ55 (lane 3). pYZ56 (lane 4) expresses a protein of increased molecular weight due to a fusion between katG and lacZ' from the vector as shown in panel

C. Panel C also shows partial sequence alignment with E. coli HPI.

Fig. 3 shows an E. coli strain with mutations in both katG and katE (UM2 Mulvey et al., 1988) that was transformed with pUC19 vector alone, pYZ55 expressing M. tuberculosis katG and pYZ56 with high level expression of M. tuberculosis katG. Overnight cultures in Luria-Bertani broth supplemented with appropriate antibiotics were plated out in the presence of varying concentrations of INH and colony forming units were assessed. Results of a representative experiment are shown with error bars indicating the standard deviation observed in triplicate samples. Overexpression of M. tuberculosis katG similarly conferred susceptibility to high concentrations of INH in E. coli UM255 (katG, katE, Mulvey et al., 1988), but had no effect on catalase-positive strains such as E. coli TG1. In some experiments, high concentrations of INH had detectable inhibitory effect on growth of UM2 and UM255, alone, but in all experiments inhibition of pYZ56-transformants was at least 10-100 fold greater than that observed in the corresponding vector controls.

Fig. 4 shows Southern blots prepared using genomic DNA from different M. tuberculosis strains, digested with Kpnl, that were probed with (A) katG (the 4.5 kb Kpnl fragment), and (B) the SOD gene (1.1 kb EcoRl-Kpnl fragment, Zhang et al., 1991). Labelling of probes and processing of blots was performed as described previously (Eiglmeier et al., 1991; Maniatis et al., 1989). Lane 1, H37Rv; 2, strain 12 - MIC 1.6 μg/ml INH; 3, B1453 - MIC > 50 μg/ml INH (Jackett et al., 1978); 4, strain 24 - MIC > 50 μg/ml INH; 5, 79112 - INH-sensitive (Mitchison et

al., 1963); 6, 12646 - INH-sensitive (Mitchison et al., 1963); 7, 79665 - INH-sensitive (Mitchinson et al., 1963). INH susceptibilities were confirmed by inoculation of Lowenstein-Jensen slopes containing differing concentrations of INH.

Fig. 5. Organization of the katG locus. The upper bar corresponds to a stretch of the M. tuberculosis chromosome spanning the katG region and the positions of individual cosmids used to construct the map are shown below together with the original shuttle cosmid pBH4 and pYZ55. The locations of some key restriction sites (B, BamHI; K, KpnI) are shown together with the approximate location of the known genetic markers: fbpB encoding the alpha or 85-B antigen (Matsuo et al., 1988); katG, catalase-peroxidase; LL105, an anonymous λgt11 clone kindly supplied by Å Andersen; MPTR, major polymorphic tandem repeat (Hermans et al., 1992).

Fig. 6. A. Nucleotide sequence of the KpnI fragment bearing katG. This sequence has been deposited in the EMBL data-library under accession number X68081. The deduced protein sequence is shown in the one letter code. B. Alignment of the two copies of the 700 bp direct repeat with identities shown as * and - denoting pads introduced to optimize the alignment. Numbering refers to the positions in Fig. 2A.

Fig. 7. Distribution of katG in mycobacteria. A. Samples of different bacterial DNAs (1.5 μg) were digested with RsrII, separated by agarose gel electrophoresis and stained with ethidium bromide; lanes 1 and 7, size markers; M. leprae; lane 3, M. tuberculosis H37Rv; lane 4, M. gordonae; lane 5, M. szulgai; lane 6, M. avium. B. Hybridization of the gel in A, after

Southern blotting, with a katG specific probe.

Fig. 8. Primary structure alignment of catalase-peroxidases. The sequences are from M. tuberculosis H37RV, mtkatg; E. coli, eckatg (Triggs-Raine et al., 1988); S. typhimurium, stkatg; B. stearothermophilus, bspera (Loprasert et al., 1988) and yeast cytochrome c peroxidase (ccp; Finzel et al., 1984). The alignment was generated using PILEUP and PRETTY (Devereux et al., 1984)

and . denote gaps introduced to maximize the homology. Key residues from the active site and the peroxidase motifs (Welinder, 1991), discussed in the text, are indicated below the consensus.

Fig. 9. Western blot analysis of M. tuberculosis KatG produced in different bacteria. Proteins were separated by SDS-polyacrylamide gel electrophoresis then subjected to immunoblotting, and detection with antiserum raised against BCG, as described in Zhang et al., 1991.

Lane 1, soluble extract of M. tuberculosis H37Rv; lane 2, M. smegmatis MC^2155 harboring the vector pBAK14; lane 3, MC^2155 harboring pBAK-KK (katG+); lane 4, E. coli UM2 (katE, katG), lane 5, UM2 harboring pYZ55 ($katG^+$); lane 6, UM2 harboring pYZ56 (lacZ'::katG).

DETAILED DESCRIPTION OF PREFERRED EMBODIMENT

The recent emergence of large numbers of strains of M. tuberculosis showing multidrug resistance in the United States is a most alarming development given the extreme contagiousness of this organism. This danger has been strikingly illustrated by several small tuberculosis epidemics in which a single patient infected with MDR M. tuberculosis has infected both HIV-positive individuals, prison guards and healthy nursing staff (CDC 1990, 1991;

Daley et al., 1992; Snider and Roper, 1992). Given the gravity of the current worldwide HIV epidemic, it is conceivable that if AIDS patients in the West, like those in Africa, were to be infected with MDR M. tuberculosis strains (rather than members of the M. avium/M. intracellulare complex) widespread dissemination of the disease would result.

Isoniazid (INH) is a bactericidal drug which is particularly potent against the tuberculosis group of mycobacteria - Mycobacterium tuberculosis, M. bovis, and M. africanum - and, in consequence, it has been particularly effective in the treatment of tuberculosis. Standard anti-tuberculosis regimens generally include INH and rifampicin, often in combination with the weaker drugs, pyrazinamide, ethambutol or streptomycin. Besides its use in therapy INH is also given to close contacts of patients as a prophylactic measure.

INH-resistant mutants of M. tuberculosis, the agent of the human disease, show two levels of resistance: low (1 to 10 μg/ml) and high (10 to 100 μg/ml). INH-resistance is often associated with loss of catalase activity and virulence. Recently, owing to the AIDS epidemic, increased homelessness and declining social conditions, tuberculosis has reemerged as a major public health problem in developed countries, particularly the USA. An alarming feature of the disease today is the emergence of multiple drug-resistant organisms and rapid nosocomial transmission to health care workers and HIV-infected patients. This has prompted CDC to propose new recommendations for the treatment of multiple resistant strains (at least INH and rifampicin) and the prevention of transmission. To obtain fresh insight into the

problem of INH-resistance and to develop a rapid diagnostic test the following study was performed.

Clearly, it is essential to understand the mechanisms of resistance to INH and rifampicin, the main anti-tuberculosis agents, as this will allow novel chemotherapeutic strategies to be developed and facilitate the design of new compounds active against MDR strains.

This invention demonstrates that it is the catalase-peroxidase enzyme, HPI, which is the INH target, and it is suggested that this enzyme alone mediates toxicity. Compelling evidence of this conclusion was obtained by expression of the M. tuberculosis katG gene in a catalase-negative mutant of E. coli as this resulted in this bacterium becoming sensitive to INH. Moreover, the isolation of the M. tuberculosis INH-sensitivity gene, katG, is important as it will facilitate the rapid detection of INH-resistant strains by means of hybridization and PCR-based approaches. The high frequency of katG deletions in clinical strains, as shown here, should simplify this procedure.

Identification of an M. tuberculosis gene involved in INH-sensitivity

A heterologous approach was employed to isolate M. tuberculosis gene(s) involved in INH-sensitivity. BH1 is a spontaneous mutant of the easily transformable M. smegmatis strain MC^2155 (Snapper et al., 1990), that is resistant to 512 μg/ml of the INH and lacks catalase-peroxidase activity (Heym et al., 1992). As there is a strict correlation between INH-sensitivity and these enzyme activities, transformation of BH1 with a

plasmid carrying the appropriate gene from M. tuberculosis should lead to their restoration and concomitant INH-sensitivity.

Consequently, DNA was prepared from a pool of M. tuberculosis shuttle cosmids in Escherichia coli and introduced into BH1 by electro-transformation. Over 1000 kanamycin-resistant transformants were then scored for INH-sensitivity, and four clones that failed to grow on medium containing 32 g/ml of INH, the MIC from wild type strain MC^2155, were obtained.

After re-transformation of BH1, only one of these, pBH4, consistently conferred the INH-sensitive phenotype. Restriction digests with BamHI, KpnI, NotI, ClaI and HindIII showed the M. tuberculosis chromosomal DNA carried by pBH4 to be about 30 kb in size. A map produced with the last three enzymes is presented in Fig. 1.

When pBH4 was used as a hybridization probe to detect homologous clones in the library, a further eight shuttle cosmids were isolated. On transformation into BH1, five of these (T35, T646, T673, T79, T556) restored INH-sensitivity, and showed similar restriction profiles to pBH4. In particular, a KpnI fragment of 4.5 kb was present in all cases.

Attempts to subclone individual BamHI fragments did not give rise to transformants capable of complementing the lesion in BH1 suggesting that a BamHI site might be located in the gene of interest. In contrast, pBH5, a derivative of pBH4, was constructed by deletion of EcoRI fragments and this showed that a 7 kb segment was not required for restoration of INH-sensitivity.

Transformants harboring shuttle cosmids that

complemented the INH-resistant mutation of BH1 were examined carefully and the MICs for several antibiotics were established. In all cases, the MIC for INH had been reduced from 512 to 8 µg/ml, a value lower than that of the sensitive strain MC^2155 (32 µg/ml). This hypersensitive phenotype suggested that the recombinant clones might be overproducing an enzyme capable of enhancing INH-toxicity. Enzymological studies showed that these transformants all produced about 2-fold more peroxidase and catalase than the wild type strain MC^2155, which is INH-sensitive.

In addition to INH, many MDR-strains of M. tuberculosis are no longer sensitive to rifampicin, streptomycin, ethambutol and pyrazinamide. To examine the possibility that there might be a relationship between resistance to INH and these compounds, the MICs of several drugs for various M. smegmatis strains and their pBH4 transformants were determined, but no differences were found.

Cloning the M. tuberculosis catalase gene

A 45-mer oligonucleotide probe was designed based on the primary sequences of highly conserved regions in the catalase-peroxidase enzymes, HPI, of E. coli (Triggs-Raine et al., 1989), and Bacillus stearothermophilus (Loprasert et al., 1988). When genomic blots of M. tuberculosis DNA were probed with this oligonucleotide, specific bands were detected in most cases. As KpnI generated a unique fragment of 4.5 kb that hybridized strongly, this enzyme was used to produce a size selected library in pUC19.

Upon screening with the oligonucleotide probe, an appropriate clone, pYZ55, was obtained. A restriction

map of the insert DNA is presented in Fig. 1 where it can be seen that this corresponds exactly to part of pBH4. Independent confirmation was also obtained by cross-hybridization.

By means of various subcloning experiments the smallest fragment expressing M. tuberculosis catalase-peroxidase activity in E. coli was found to be a 2.5 kb EcoRV-KpnI fragment which, as expected, contained a cleavage site for BamHI. Partial DNA sequence analysis showed that the katG gene carried by pYZ56 encodes a catalase-peroxidase enzyme that is highly homologous to the HPI enzymes of E. coli and B. stearothermophilus:

```
M.tuberculosis      APLNSWPDNASLDKARRLLWPSKKKYGKKLSWADLIV
E.coli              *********V***********I*Q***Q*I*****FI
B.stearothermophilus*********N******C*GR**RNT*T*-*LGPICS
```

(Fig. 2; Triggs-Raine et al., 1988); (Loprasert et al., 1988). Identical residues are indicated by *. HPI activity was detected in both E. coli and M. smegmatis by staining (see below).

Catalase-peroxidase involvement in INH-sensitivity

Having cloned the M. tuberculosis katG gene, it was of immediate interest to investigate the genetic basis of the association between catalase-negativity and isoniazid resistance. A series of constructs was established in the shuttle vector pBAK14 and used to transform the INH-resistant M. smegmatis mutant BH1. Only those plasmids carrying a complete katG gene produced HPI and restored INH-sensitivity. The smallest of these, pBAK14, carried a 2.5 kb EcoRV-KpnI fragment thus demonstrating that the 2 kb region upstream of katG was not involved, and that catalase-peroxidase activity alone was

sufficient to render mycobacteria susceptible to INH.

Cell-free extracts were separated by non-denaturating polyacrylamide gel electrophoresis and stained for peroxidase and catalase activity. Under these conditions, the M. tuberculosis enzyme gave two bands of peroxidase activity (lane 1) which comigrated with catalase activity (Heym et al., 1992).

When introduced into E. coli, the katG gene directed the synthesis of the same proteins, whereas pYZ56 produced proteins slightly larger in size. This is due to the construction of an in-frame lacZ::katG gene fusion. Activity stains were also performed with cell extracts of M. smegmatis. The presence of the katG gene from the M. tuberculosis in BH1 led to the production of catalase-peroxidase enzyme, which displayed the same electrophoretic mobility as the enzyme made in M. tuberculosis, or in E. coli, and the native HPI of M. smegmatis.

Basis of INH-resistance in M. tuberculosis

It has been known for many years that a subset of INH--resistant strains, particularly those resistant to the highest drug concentrations, are of lower virulence in the guinea pig and devoid of catalase activity. Genomic DNA was prepared from several clinical isolates of M. tuberculosis and analyzed by Southern blotting using the 4.5 kb KpnI fragment as a probe. In two highly resistant strains, B1453 and 24, the catalase gene has been deleted from the chromosome whereas in others (Fig. 3), such as strain 12, showing low level resistance it is still present but not expressed. Additional studies showed that the region immediately prior to katG was highly

prone to rearrangements.

M. tuberculosis HPI renders E. coli sensitive to INH

To determine whether the HPI enzyme of M. tuberculosis could confer INH sensitivity on E. coli, a series of catalase mutants was transformed with pYZ56 and the MICs determined. Wild type strains were not susceptible to INH, but mutants lacking both endogenous catalase activities, but harboring pYZ56, showed growth inhibition when high levels of INH (500 μg/ml) were present, whereas untransformed strains were insensitive.

For purposes of this invention, a plasmid containing the restriction endonuclease map shown in Fig. 1 was deposited in strain with the National Collection of Cultures of Microorganisms (C.N.C.M.) of the Institut Pasteur, in Paris, France on May 18, 1992, under culture collection accession No. I-1209. This plasmid contains the nucleic acid sequence of the invention, namely, the 4.5 kb KpnI-KpnI fragment of plasmid pYZ56 having the BamHI cleavage ~~site in the~~ fragment.

In general, the invention features a method of detecting the presence of isoniazid-resistant Mycobacterium tuberculosis in a sample including providing at least one DNA or RNA probe capable of selectively hybridizing to isoniazid-sensitive Mycobacterium tuberculosis DNA to form detectable complexes. Detection is carried out with a sample under conditions which allow the probe to hybridize to isoniazid-sensitive Mycobacterium tuberculosis DNA present in the sample to form hybrid complexes and detecting the hybrid complexes as an indication of the presence of isoniazid-sensitive Mycobacterium

tuberculosis in the sample. (The term "selectively hybridizing", as used herein, refers to a DNA or RNA probe which hybridizes only to isoniazid-sensitive Mycobacterium tuberculosis and not to isoniazid-insensitive Mycobacterium tuberculosis.) The sample can be comprised of the Mycobacterium tuberculosis cells or a portion of the cells or cell contents enriched in Mycobacterium tuberculosis nucleic acids, especially DNA. Hybridization can be carried out using conventional hybridization reagents. The particular hybridization conditions have not been found to be critical to the invention.

More particularly, DNA sequences from Mycobacterium tuberculosis can be analyzed by Southern blotting and hybridization. The techniques used for the present invention are described in Maniatis et al. (1989). DNA fragments can be separated on agarose gels and denatured in situ. The fragments can then be transferred from the gel to a water insoluble solid, porous support, such as a nitrocellulose filter, a nylon membrane, or an activated cellulose paper, where they are immobilized for example, the Hybond® membrane commercialized by Amersham can be used. After prehybridization to reduce non-specific hybridization with the probe, the solid support is hybridized to the nucleic acid probe of the invention. The solid support is washed to remove unbound and weakly binding probe, and the resulting hybrid duplex molecule is examined. A convenient alternative approach is to hybridize oligonucleotides to the DNA denatured in the gel.

The amount of labeled probe which is present in the hybridization solution will vary widely, depending upon

the nature of the label, the amount of the labeled probe which can reasonably bind to the filter, and the stringency of the hybridization. Generally, substantial excesses of the probe over stoichiometric will be employed to enhance the rate of binding of the probe to the fixed DNA.

Various degrees of stringency of hybridization can be employed. The more severe the conditions, the greater the complementarity that is required for hybridization between the probe and the polynucleotide for duplex formation. Severity can be controlled by temperature, probe concentration, probe length, ionic strength, time, and the like. Conveniently, the stringency of hybridization is varied by changing the polarity of the reactant solution. Temperatures to be employed can be empirically determined or determined from well known formulas developed for this purpose.

Unlike Southern hybridization where DNA fragments are transferred from an agarose gel to a solid support, the method of the invention can also be carried out by oligonucleotide hybridization in dried agarose gels. In this procedure, the agarose gel is dried and hybridization is carried out in situ using an oligonucleotide probe of the invention. This procedure is preferred where speed of detection and sensitivity may be desirable. The procedure can be carried out on agarose gels containing genomic or cloned DNA of Mycobacterium tuberculosis.

In addition, the method of this invention can be carried out by transfer of Mycobacterium tuberculosis DNA from polyacrylamide gels to nylon filters by electroblotting. Electroblotting may be desirable where

time is of the essence, because electroblotting is typically faster than capillary blotting developed to transfer DNA from agarose gels. This method can be carried out in conjunction with UV-crosslinking. The polyacrylamide gel containing the samples to be tested is placed in contact with an appropriately prepared nylon filter. These are then sandwiched into an electroblotting apparatus and the DNA is transferred from the gel onto the filter using electric current. After a buffer rinse, the filter is ready to be prehybridized and hybridized or UV-crosslinked.

The method of the invention can be carried out using the nucleic acid probe of the invention for detecting Mycobacterium tuberculosis resistant to isoniazid. The probe can be detected using conventional techniques.

The method of the invention can also detect point mutations in the KatG gene, as well as a partial deletion of that gene.

The nucleotides of the invention can be used as probes for the detection of a nucleotide sequence in a biological sample of M. tuberculosis. The polynucleotide probe can be labeled with an atom or inorganic radical, most commonly using a radionuclide, but also perhaps with a heavy metal. Radioactive labels include ^{32}P, ^{3}H, ^{14}C, or the like. Any radioactive label can be employed, which provides for an adequate signal and has sufficient half-life. Other labels include ligands that can serve as a specific binding member to a labeled antibody, fluorescers, chemiluminescers, enzymes, antibodies which can serve as a specific binding pair member for a labeled ligand, and the like. The choice of the label will be governed by the effect of the label on the rate of

hybridization and binding of the probe to the DNA or RNA. It will be necessary that the label provide sufficient sensitivity to detect the amount of DNA or RNA available for hybridization.

In preferred embodiments of the invention, the probe is labeled with a radioactive isotope, e.g., ^{32}P or ^{125}I, which can be incorporated into the probe, e.g., by nick-translation.

In other preferred embodiments, the probe is labeled with biotin, which reacts with avidin to which is bonded a chemical entity which, when the avidin is bonded to the biotin, renders the hybrid DNA complex capable of being detected, e.g., a fluorophore, which renders the hybrid DNA complex detectable fluorometrically; an electron-dense compound capable of rendering the hybrid DNA complexes detectable by an electron microscope; an antibody capable of rendering the hybrid DNA complexes immunologically detectable; or one of a catalyst/substrate pair capable of rendering the hybrid DNA complexes enzymatically detectable. Prior to contacting the bacteria with the probe, the M. tuberculosis bacteria can be lysed to release their DNA, which is then denatured and immobilized on an appropriate solid, DNA-binding support, such as a nitrocellulose membrane.

Another detection method, which does not require the labeling of the probe, is the so-called sandwich hybridization technique. In this assay, an unlabeled probe, contained in a single-stranded vector, hybridizes to isoniazid-sensitive Mycobacterium tuberculosis DNA, and a labeled, single-stranded vector, not containing the probe, hybridizes to the probe-containing vector, labeling the whole hybrid complex.

The sequences of the invention were derived by dideoxynucleotide sequencing. The base sequences of the nucleotides are written in the 5'-----> 3' direction. Each of the letters shown is a conventional designation for the following nucleotides:

A Adenine

G Guanine

T Thymine

C Cytosine.

The nucleotides of the invention can be prepared by the formation of 3'-----> 5' phosphate linkages between nucleoside units using conventional chemical synthesis techniques. For example, the well-known phosphodiester, phosphotriester, and phosphite triester techniques, as well as known modifications of these approaches, can be employed. Deoxyribonucleotides can be prepared with automatic synthesis machines, such as those based on the phosphoramidite approach. Oligo- and polyribonucleotides can also be obtained with the aid of RNA ligase using conventional techniques.

The nucleotides of the invention are in a purified form. For instance, the nucleotides are free of human blood-derived proteins, human serum proteins, viral proteins, nucleotide sequences encoding these proteins, human tissue, and human tissue components. In addition, it is preferred that the nucleotides are free of other nucleic acids, extraneous proteins and lipids, and adventitious microorganisms, such as bacteria and viruses.

This invention of course includes variants of the nucleotide sequences of the invention or serotypic variants of the probes of the invention exhibiting the same selective hybridization properties as the probes

identical herein.

The nucleotide sequences of the present invention can be employed in a DNA amplification process known as the polymerase chain reaction (PCR). See, e.g., Kwok et al. (1987). PCR is advantageous because this technique is rapid.

DNA primer pairs of known sequence positioned 10-300 base pairs apart that are complementary to the plus and minus strands of the DNA to be amplified can be prepared by well known techniques for the synthesis of oligonucleotides. One end of each primer can be extended and modified to create restriction endonuclease sites when the primer is annealed to the PBMC DNA. The PCR reaction mixture can contain the PBMC DNA, the DNA primer pairs, four deoxyribonucleoside triphosphates, $MgCl_2$, DNA polymerase, and conventional buffers. The DNA can be amplified for a number of cycles. It is generally possible to increase the sensitivity of detection by using a multiplicity of cycles, each cycle consisting of a short period of denaturation of the PBMC DNA at an elevated temperature, cooling of the reaction mixture, and polymerization with the DNA polymerase.

Amplified sequences can be detected by the use of a technique termed oligomer restriction (OR). Single-strand conformation polymorphism (SSCP) analysis can be used to detect DNA polymorphisms and point mutations in a variety of positions in DNA fragments. See, Saiki et al. (1985); Orita et al. (1989). For example, after amplification, a portion of the PCR reaction mixture can be separated and subjected to hybridization with an end-labeled nucleotide probe, such as a ^{32}p labelled adenosine triphosphate end-labeled

probe. In OR, an end-labeled oligonucleotide probe hybridizes in solution to a region of the amplified sequence and, in the process, reconstitutes a specific endonuclease site. Thus, hybridization of the labeled probe with the amplified katG sequence yields a double-stranded DNA form that is sensitive to selective restriction enzyme digestion. After restriction with an endonuclease, the resulting samples can be analyzed on a polyacrylamide gel, and autoradiograms of the portion of the gel with the diagnostic labeled fragment can be obtained. The appearance of a diagnostic fragment (e.g., 10-15 bases in length) in the autoradiogram indicates the presence of katG sequences in the PBMCs.

Since it may be possible to increase the sensitivity of detection by using RNA instead of chromosomal DNA as the original template, this invention contemplates using RNA sequences that are complementary to the DNA sequences described herein. The RNA can be converted to complementary DNA with reverse transcriptase and then subjected to DNA amplification.

EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids

Table 1 outlines the properties of the bacterial strains and plasmids used in this invention.

Table 1. Bacterial Strains And Plasmids

Strains/plasmids	Characteristics
E. coli NM554	
E. coli TG1	*supE hsd5 thi delta (lac-proAB)* [*traD36 proAB+ lacI*^{q} *lacZ delta M15*]
E. coli UM2	KatE
E. coli UM255	KatE
M. tuberculosis H37Rv	Virulent strain originally isolated from tuberculosis patient
M. tuberculosis 12	Clinical isolate resistant to low levels of INH (1-2 μg/ml)
M. tuberculosis B1453	Clinical isolate resistant to high levels of INH (>50 μg/ml)
M. tuberculosis 24	Clinical isolate resistant to high levels of INH (>50 μg/ml)
M. tuberculosis 79112	Clinical isolate sensitive to INH
M. tuberculosis 12646	Clinical isolate sensitive to INH
M. tuberculosis 79665	Clinical isolate sensitive to INH
M. smegmatis MC^{2}155	MC^{2}6 *het*
M. smegmatis BH1	MC^{2}155 *het katG*

Plasmids

pBH4	Shuttle cosmid, katG+, based on pYUB18
pBH5	Deleted version of pBH4, katG+, (7 kb-EcoRI)
pYZ55	pUC19 derivative with 4.5 kb KpnI fragment, kat+
pYZ56	pUC19 derivative with 2.5 kb EcoRV-KpnI fragment (kat+)
pYZ57	pUC19 derivative with 3.1 kb KpnI-BamHI fragment, kat-
pBAK14	Mycobacterial shuttle vector (Zhang et al., 1991)
pBAK15	Mycobacterial shuttle vector carrying 4.5 kb KpnI fragment (kat+)
pBAK16	Mycobacterial shuttle vector carrying 2.5 kb EcoRV-KpnI fragment (kat^+)
pBAK17	Mycobacterial shuttle vector carrying 3.1 kb KpnI-BamHI fragment (kat-)

The *M. tuberculosis* H37 RV genomic library was constructed in the shuttle cosmid pYUB18 (Snapper et al., 1988) and kindly supplied by Dr. W. R. Jacobs. Other shuttle vectors employed were pYUB12 (Snapper et al., 1988) and pBAK14 (Zhang et al., 1991).

Microbiological techniques and enzymology

Details of antibiotics used, growth conditions, enzymology and MIC determinations can be found in Heym et al., (1992).

Nucleic acid techniques

Standard protocols were used for subcloning, Southern blotting, DNA sequencing, oligonucleotide biosynthesis, etc. (Maniatis et al., 1989; Eiglmeier et al., 1991).

Activity staining

The preparation of cell-free extracts of E. coli and mycobacteria has been described (Heym et al., 1992; Zhang et al., 1991). Native protein samples were separated by polyacrylamide gel electrophoresis as described by Laemmli (1970) except that SDS was omitted from all buffers, samples were not boiled and betamercaptoethanol was not included in the sample buffer. After electrophoresis of 50 - 100 µg protein samples on 7.5% polyacrylamide gels, catalase activity was detected by soaking the gel in 3mM H_2O_2 for 20 minutes with gentle shaking. An equal volume of 2% ferric chloride and 2% potassium ferricyanide was added and clear bands of catalase activity revealed by illumination with light. Peroxidase activity was detected as brown bands after soaking gels in a solution containing 0.2 - 0.5 mg/ml diaminobenzidine and 1.5 mM H_2O_2 for 30 - 120 minutes.

To generate a highly toxic compound it seems most likely that the M. tuberculosis HPI enzyme peroxidatively activates INH (Youatt, 1969; Gayathri-Devi et al., 1975). Now that the katG gene has been isolated and characterized, it should be possible to make new derivatives of INH, which can be activated in a similar manner.

Example 1

Point mutations in the katG gene associated with the isoniazid-resistance of M. tuberculosis

It has been shown in a recent study that the catalase-peroxidase of Mycobacterium tuberculosis, encoded by the katG gene, is involved in mediating the toxicity of the potent anti-tuberculosis drug isoniazid or INH. Mutants resistant to clinical levels of INH show reduced catalase-peroxidase activity and, in some cases, this results from the deletion of the katG gene from the chromosome.

Transformation of INH-resistant strains of Mycobacterium smegmatis and M. tuberculosis with the cloned katG gene leads to restoration of drug-sensitivity. Expression of katG in some strains of Escherichia coli renders this naturally resistant organism susceptible to high concentrations of INH.

As some INH-resistant clinical isolates of M. tuberculosis have retained an intact katG gene, the molecular basis of their resistance was investigated. This study was facilitated by the availability of the nucleotide sequence of a 4.7 kb KpnI fragment from the katG region of the chromosome as this allowed primers suitable for PCR analysis to be designed. Eleven pairs of oligonucleotide primers were synthesized (see Table 2) and used to generate PCR-products, of around 280 bp, that covered the complete katG gene and some of the flanking sequences. In control experiments all experiments all eleven primer pairs generated PCR products of the expected size, highly suitable for SSCP-analysis, so a panel of 36 INH-resistant strains of M. tuberculosis, of Dutch or French origin, was examined. Many of these strains are multidrug resistant and were isolated from patients who were HIV-seropositive.

Table 2. Sequences of primer pairs used for PCR-SSCP analysis of the *katG* gene of *M.tuberculosis*

		5'	3'	Length	G+C(%)	Tm	Production
Primer Pair #	1						
OLIGO1:	GCGGGGTTATCGCCGATG	1765	1782	18	66	61.8	288
OLIGO2:	GCCCTCGACGGGGTATTTC	2052	2034	19	63	61.9	
Primer Pair #	2						
OLIGO1:	AACGGCTGTCCCGTCGTG	2008	2025	18	66	61.9	300
OLIGO2:	GTCGTGGATGCGGTAGGTG	2307	2289	19	63	61.9	
Primer Pair #	3						
OLIGO1:	TCGACTTGACGCCCTGACG	2169	2187	19	63	61.9	280
OLIGO2:	CAGGTCCGCCCATGACAG	2448	2431	18	66	61.9	
Primer Pair #	4						
OLIGO1:	CCACAACGCCAGCTTCGAC	2364	2382	19	53	61.9	284
OLIGO2:	GGTTCACGTAGATCAGCCCC	2647	2628	20	50	61.9	
Primer Pair #	5						
OLIGO1:	GCAGATGGGGCTGATCTACG	2622	2641	20	60	51.9	288
OLIGO2:	ACCTCGATGCCGCTGGTG	2909	2892	18	66	51.9	
Primer Pair #	6						
OLIGO1:	GCTGGAGCAGATGGGCTTG	2829	2847	19	63	61.9	286
OLIGO2:	ATCCACCCGCAGCGAGAG	3114	3097	18	66	61.9	
Primer Pair #	7						
OLIGO1:	GCCACTGACCTCTCGCTG	3088	3105	18	66	61.9	297
OLIGO2:	CGCCCATGCGGTCGAAAC	3384	3367	18	66	61.9	

Primer Pair #	8					61.9	285
OLIGO1:	GCGAAGCAGATTGCCAGCC	3304	3322	19	63	61.9	
OLIGO2:	ACAGCCACCGAGCACGAC	3588	3571	18	66		
Primer Pair #	9					61.9	281
OLIGO1:	CAAACTGTCCTTCGCCGACC	3549	3568	20	60	61.9	
OLIGO2:	CACCTACCAGCACCGTCATC	3829	3810	20	60		
Primer Pair #	10					61.9	280
OLIGO1:	TGCTCGACAACGCGAACCTG	3770	3789	20	60	61.9	
OLIGO2:	TCCGAGTTGGACCCGAAGAC	4049	4030	20	60		
Primer Pair #	11					61.9	280
OLIGO1:	TACCAGGGCAAGGATGGCAG	3973	3992	20	60	61.9	
OLIGO2:	GCAAACACCAGCACCCCG	4252	4235	18	66		

{#courier10}

Two of them gave no PCR fragment, with any of the primers used, indicating that katG had been deleted. The remaining 34 strains all yielded the expected PCR products and these were analyzed on SSCP gels so that possible point mutations could be

detected. In 20 cases, abnormal strand mobility was observed, compared to that of katG from drug-sensitive *M. tuberculosis*, suggesting that mutational events had indeed occurred. The approximate locations of the mutations, as delimited by the PCR primers, are shown in Table 3.

Table 3. Preliminary results of PCT-SSCP analysis of *katG* from *M. tuberculosis* strains x denotes altered mobility; del denotes deletion

N°	Strain	MIC (INH)	1 1765-2034	2 2008-2289	3 2169-2431	4 2364-2628	5 2622-2892	6 2829-3097	7 3088 3367
1/37	9488	1							X
2	9577	1							
3	9112	10							
4	9247	1							
5	9200	1					X		
6	9116	1							
7/31	9106	1					X		X
8	9291	1							X
9/10	9412	1	X						
11/12	9435	1							
13	9428	1			X				
14	9441	1			X				X
15/16	9444	1			X				X
17/18	9445	1							
19/20	9330	0,2			X				
21/22	9420	0,2							
23	9262	0,2							
24/38	9523	1					X		
25	9592	10			X				
26	9553	10							
27	9485	10					X		
28	9181	1			X		X		
29	9363	1							X
30	9465	1			X				
32	9178	0,2							
33	9468	0,2							
34	9218	0,2							

N°	Strain	MIC (INH)	1 1765-2034	2 2008-2289	3 2169-2431	4 2364-2628	5 2622-2892	6 2829-3097	7 3088 3367
33	9468	0,2							
34	9218	0,2							
35	9503	0,2					X		
39	9582	1							
41	H37Rv	-							
42	Ass	-							
43	Mou	-							
44	13632	>20	del	del	del	del	del	del	del
45	13549	>5	del	del	del	del	del	del	del
46	13749	>20							X
47	14006	10							
48	13711	>5					X		
49	13681	>5							
50	14252	>5							

On examination of a 200 bp segment of the katG gene from five independent strains (9188, 9106, 9441, 9444, 9363), a single base difference was found. This was the same in all cases, a G to T transversion at position 3360, resulting in the substitution of Arg-461 by Leu. Thus, in addition to inactivation of katG, INH-resistance can stem from mis-sense mutations that result in an altered catalase peroxidase. This mutation may define a site of interaction between the drug and the enzyme. The results of DNA sequence studies with the remaining mutants are eagerly awaited.

Another conclusion that can be drawn from this study concerns the molecular basis of the multidrug resistance associated with various M. tuberculosis strains. The same mutations are found irrespective of whether a given patient is seropositive or seronegative for HIV. For example, strain 9291, isolated from an HIV-seropositive tuberculosis patient, harbors mutations conferring resistance to INH, rifampin and streptomycin in the katG (R461L), rpoB (S425L) and rpsL (K42R) genes, respectively. The same mutations have been found separately, or in combination, in strains from HIV-seronegative individuals. This means that, for the set of strains studied, there is no novel, single mechanism conferring resistance to several drugs, but rather,

multidrug resistance results from the accumulation of mutations in the genes for distinct drug targets.

Example 2

Nucleotide sequence and chromosomal location of the katG locus of M. tuberculosis

Bacterial strains, plasmids and growth conditions. The following bacterial strains from our laboratory collections were used in this study: M. tuberculosis H37Rv; M. smegmatis MC^2155 (Snapper et al., 1990); E. coli K-12 UM2 (katE katG; Mulvey et al., 1988). The recombinant plasmids, pYZ55 (pUC19, $katG^+$), pYZ56 (pUC19, lacZ'::katG) and the shuttle clones, pBH4 (pYUB18, $katG^+$) and pBAK-KK- (pBAK14, $katG^+$) have been described recently (Zhang et al. 1992, Nature) and the katG locus of M. tuberculosis is schematized in Fig. 5. Mycobacteria were grown at 37°C in Middlebrook 7H9 medium, while E. coli strains were cultivated in L-broth, with appropriate enrichments and antibiotics.

Nucleic acid techniques. Standard techniques were employed for the preparation, labelling and hybridization of DNA (Eiglmeier et al. 1991; Zhang et al. 1992, Infect. Immun.; Zhang et al. 1992, Nature). A shotgun library of random fragments of pYZ55 was prepared in M13mp18 as described previously (Garnier et al., 1986) and sequenced using the modified dideoxy technique (Biggin et al. 1983). Sequences were compiled and assembled into contigs using SAP, and analyzed with NIP, SIP and PIP (Staden 1987) running on a Vax 3100 workstation. Gap closure was obtained by using synthetic oligonucleotide primers, synthesized on an ABI 381 apparatus, and T7 DNA polymerase (Pharmacia) to obtain sequences directly from pYZ55. To search for related sequences in the GenBank database (release 73.1) the FASTA (Pearson et al. 1988) and BLAST (Altschul et al. 1990)

programs were used. The PROSITE (Bairoch 1992) catalog was screened to detect possible motifs present in protein sequences and alignments were done with the PILEUP and PRETTY modules of the GCG sequence analysis package (Devereux et al. 1984).

Western blotting and catalase-peroxidase activity staining. Immunoblotting of polypeptides resolved by SDS-polyacrylamide gel electrophoresis and detection with polyclonal antibodies (purchased from DAKO) raised against M. bovis BCG, were as described (Zhang et al. 1992, Infect. Immun., Nature, Mol. Microbiol.). Procedures for detecting catalase and peroxidase activities have been outlined recently (Heym et al. 1992; Zhang et al. 1992, Nature).

RESULTS

Nucleotide sequence of the katG locus of M. tuberculosis. In previous studies, the complete katG gene was cloned independently in E. coli on a shuttle cosmid, pBH4, and on a 4.5 kb KpnI restriction fragment thus giving rise to pYZ55 (Fig. 5; Zhang et al. 1992, Nature). The structural gene for catalase-peroxidase was subsequently localized to a 2.5 kb EcoRV - KpnI fragment by sub-cloning. To deduce the primary structure of this important enzyme and thereby gain some insight into its putative role in the conversion of INH into a potent anti-tuberculous derivative, the nucleotide sequence of the complete insert from pYZ55 was determined. This was achieved by the modified dideoxy-shotgun cloning procedure (Biggin et al. 1993) and gaps between the contigs were closed by using specific primers.

On inspection of the resultant sequence which is shown in Fig. 6A, the 4.5 kb fragment was found to contain 4795 nucleotides with an overall dG+dC content of 64.4%. When this was analyzed for the presence of open reading frames,

with high coding-probability values, a single candidate was detected and, from its size, composition and location, this was identified as katG. The absence of any additional open reading frames, on either strand of the KpnI fragment, ruled out the possibility that genes other than katG were involved in conferring INH-susceptibility.

Further analysis of the sequence showed katG to be preceded by two copies of a 700 bp direct repeat which were 68% identical, with the longest stretch of identity comprising 58 bp (Fig. 6B). When the databases were screened with this sequence no significant homologies were detected. To test the possibility that it could correspond to a new repetitive element in M. tuberculosis, a 336 bp probe, encompassing the 58 bp repeat, was used to probe a partially-ordered cosmid library. Positive hybridization signals were only obtained from clones that were known to carry katG. Likewise, a single restriction fragment was detected in Southern blots of M. tuberculosis DNA digested with restriction enzymes BamHI, KpnI and RsrII thereby indicating that this repetitive sequence is not dispersed.

Chromosomal location of katG. As part of the M. tuberculosis genome project, most of the genes for which probes are available have been positioned on the contig map. From the series of overlapping cosmids shown in Fig. 5 it can be seen that the markers linked to katG are LL105 and fbpB encoding an anonymous antigen and the putative fibronectin binding protein, or alpha antigen (Matsuo et al. 1988), respectively. None of the known insertion sequences IS6110 and IS1081 (Collins et al. 1991; McAdam et al. 1990; Thierry et al. 1990, J. Clin. Microbiol.; Thierry et al. 1990, Nucleic Acids Res.), map to this area of the chromosome although the region upstream of katG is densely populated

with copies of the major polymorphic tandem repeat, MPTR (Hermans et al. 1992; Zhang and Young 1993).

Presence of katG homologues in other mycobacteria. INH is exquisitely potent against members of the tuberculosis complex yet shows little, if any, activity against other mycobacteria. To determine whether genes homologous to katG were present in other mycobacteria Southern blots of DNA digested with RsrII were hybridized with a probe prepared from a 2.5 kb EcoRV-KpnI restriction fragment carrying katG from M. tuberculosis. Under conditions of high stringency good signals were obtained from M. leprae and M. avium (Fig. 7) while barely discernible hybridization was observed with M. gordonae and M. szulgai. It has been shown recently that katG homologues are also present in M. Smegmatis and M. aurum (Heym et al. 1992).

Predicted properties of catalase-peroxidase from M. tuberculosis. The primary structure of catalase-peroxidase, deduced from the nucleotide sequence of katG, is shown in Fig. 6. The enzyme is predicted to contain 735 amino acids, and to display a molecular weight of 80,029 daltons. A protein of this size has been observed in M. tuberculosis, and both recombinant M. smegmatis and E. coli (see below).

Primary structures are available for several other bacterial catalase-peroxidases including those from E. coli, salmonella typhimurium and Bacillus stearothermophilus (Loewen et al. 1990; Loprasert et al. 1988; Triggs-Raine et al. 1988) and these have been shown to be distantly related to yeast cytochrome c peroxidase (Welinder 1991). As the crystal structure of the latter has been determined (Finzel et al. 1984) this can be used to interpret the sequences of the bacterial enzymes. The M. tuberculosis enzyme shows 53.3% conservation with the enterobacterial HPI enzymes, and

shares 45.7% identity with the protein from B. stearothermophilus. An alignment of the sequences of these four enzymes is shown in Fig. 8, along with that of yeast cytochrome c peroxidase (Welinder 1991). It is apparent that the NH_2 terminus, which has no counterpart in the yeast enzyme, is the most divergent part suggesting that this domain of the protein can tolerate extensive deviation and is not required for catalysis. Experimental support for this interpretation is provided in the form of a LacZ-KatG fusion protein which contains an additional 40 amino acid residues (Fig. 9, lane 6; Zhang et al. 1992, Nature). Addition of this NH_2-terminal segment does not noticeably interfere with either the catalase or peroxidase reactions effected by KatG as judged by activity staining (Zhang et al. 1992, Nature).

Bacterial catalase-peroxidases are believed to have evolved by means of a gene duplication event and consist of two modules, both showing homology to the yeast enzyme, fused to a unique NH_2-terminal sequence of about 50 amino acid residues (Welinder 1991). The M. tuberculosis enzyme conforms to this pattern and when searched for internal homology using SIP (Staden 1987) it was clear that the region between residues 55-422 was related to the carboxy terminal domain, consisting of amino acids 423-735. Only one of the two active site motifs typical of peroxidases, present in the PROSITE catalog (Bairoch 1992) was found when the M. tuberculosis catalase-peroxidase primary structure was screened as there are two deviations from the consensus around His^{269} where the second motif should be. (Consensus pattern for peroxidase 1: [DET]-[LIVMT]-x(2)-[LIVM]-[LIVMSTAG]-[SAG]-[LIVMSTAG]-H-[STA]-[LIVMFY]; consensus pattern for peroxidase 2: [SGAT]-x(3)-[LIVMA]-R-[LIVMA]-x-[FW]-H-x-[SAC]; (Bairoch

1992). In addition, a possible ATP-binding motif (G-x-x-x-x-G-K-T) was detected (Balroch 1992) but as this partially overlaps the active site its presence may be purely fortuitous (Fig. 8).

By analogy with yeast cytochrome c peroxidase (Welinder 1991), it was possible to predict a number of structurally and catalytically important residues all of which are located in the NH_2-terminal repeat. His^{269} should serve as the fifth ligand of the heme-iron while Asp^{380} should be its hydrogen-bonded partner. Other residues predicted to be involved in active site modulation and H_2O_2 binding are Arg^{104}, Trp^{107}, His^{108}, Asn^{138}, Thr^{274} and His^{275} (Fig. 4). According to Welinder's predictions (Welinder 1991), Trp^{320} should be a key residue and be required for forming the protein-radical site (Sivaraja et al. 1989).

Antibody response to M. tuberculosis KatG. To evaluate the possible value of KatG as an immunogen, Western blots were probed with anti-serum raised against M. bovis BCG in rabbits. As shown in Fig. 9, the 80 kD catalase-peroxidase is one of the prominent antigens recognized in cell-free extracts of M. tuberculosis, and M. smegmatis expressing the cloned katG gene (lanes 1, 3). Likewise, on introduction of the gene into E. coli significant levels of catalase-peroxidase were produced a striking increase in expression was obtained from the lacZ'-katG gene fusion which directed the synthesis of an 85 kD fusion protein (Fig. 9, lane 6).

The aim of the present study was to determine the nucleotide sequence of the katG gene and to use the information obtained to try and understand how its product mediates the INH-susceptibility of M. tuberculosis and, possibly, to explain the apparent instability of the katG

region of the genome. Repetitive DNA is often a source of chromosomal rearrangements and analysis of the DNA sequence upstream of katG revealed two copies of a 700 bp direct repeat. Since this element appears to be confined to this locus it is unlikely to serve as a target for an event, such as homologous recombination, which could lead to the deletion of the gene that is observed so frequently (Zhang et al. 1992, Nature; Zhang and Young 1993). Likewise, as a 70 kb stretch of the chromosome of M. tuberculosis H37Rv, encompassing katG, is devoid of copies of IS6110 and IS1081, these insertion sequences do not appear to be likely sources of instability. Rather, the presence of a cluster of major polymorphic tandem repeats, MPTR (Fig. 5; Hermans et al. 1992) situated upstream of katG, suggests that this might act as a recombinational hotspot. It may remove both the MPTR cluster and katG (Zhang and Young 1993). The availability of the sequence of the katG region will allow primers suitable for the polymerase chain reaction to be designed and thus facilitate studies aimed at both rapid detection of INH-resistance and understanding the molecular basis of chromosomal instability.

Perhaps the most intriguing feature of the M. tuberculosis catalase-peroxidase is its ability to mediate INH-susceptibility. In our current working hypothesis, the drug interacts with the enzyme and is converted by the peroxidase activity into a toxic derivative which acts at a second, as yet unknown, site (Zhang et al. 1992, Nature). Although horse radish peroxidase can effect this reaction (Pearson et al. 1988; Shoeb et al. 1985), and produce hydroxyl and organic free radicals, very few bacteria, including other mycobacteria, are sensitive to INH. This is intriguing as they contain genes homologous to katG (Fig. 7).

One explanation for this could be provided by the fact that most bacterial contain two catalases, one of which is a broad spectrum enzyme endowed with peroxidase activity, and that the second catalase, by preferentially removing H_2O_2, limits the ability of the catalase-peroxidase to oxidize INH. As M. tuberculosis lacks the latter activity its KatG enzyme can convert INH to the lethal form without competition for the electron acceptor.

Alternatively, there may be some unique features of the M. tuberculosis enzyme which promote toxicity or favor the interaction with the drug. Examination of the primary structures of the bacterial catalase-peroxidases was not instructive in this respect as they all share extensive sequence identities and contain two motifs characteristic of the active sites of peroxidases. Furthermore, it has been shown recently that expression of the E. coli katG gene can partially restore INH-susceptibility to drug-resistant mutants of M. tuberculosis suggesting that the endogenous enzyme may not possess any drug-specific properties (Zhang et al. 1993). Sequence comparison with the cytochrome c peroxidase from yeast has provided important information about the structural and functional organization of the KatG protein and led to the identification of the putatively-important catalytic residues (Fig. 8).

Now that the complete sequence of katG is available it will be possible to test some of these hypotheses by site-directed mutagenesis and to overproduce the enzyme so that detailed analysis of the enzymatic reaction, and its products, can be performed in vitro. Likewise, it should be a relatively simple matter to isolate mutants that have retained enzymatic activity but are unable to bind or oxidize INH. Of particular interest is the repetitive structure of

the enzyme and the prediction that the NH_2-terminal repeat contains the active site for peroxidases. This raises the possibility that katG genes, mutated, or truncated at the 3'-end, could arise. It is conceivable that their products, lacking the normal COOH-terminus which may be required for subunit-subunit interactions (Welinder 1991), would be unstable but still retain low enzyme activity. They would thus confer an intermediate level of INH-susceptibility, between that of katG⁺ strains and mutants completely lacking the gene, as is often observed in clinical settings.

The invention may of course make use of a part of the above described 2.5 kb EcoRV-KpnI fragment, said part being nonetheless sufficiently long to provide for the selectivity of the *in vitro* detection of a Mycobacterium tuberculosis resistant to isoniazid.

The invention also relates to a kit for detecting multidrug resistant variants of *M. tuberculosis* wherein the kit comprises:

(a) a container means containing a probe for the gene encoding drug ~~resistance;~~ and

(b) a container means containing a control preparation of nucleic acid.

Needless to say that use can be made of any detection method alternative bringing into play the nucleodic sequence specific of nucleic acids of a Mycobacterium resistant to isoniazid, e.g. a method using an amplification technique and primers, whereby said primers may either be contained within said specific nucleotidic sequence, in order to provide for amplification fragments containing at least a part of the nucleotide sequence of the above mentioned probe, nonetheless sufficiently long to provide for the selectivity of the *in vitro* detection of a Mycobacterium tuberculosis resistant to

isoniazid, and finally detecting a possible mutation in any of the amplified sequences.

A preferred process alternative (oligotyping) for the detection of resistance to the selected antibiotic comprises:

- fragmenting the relevant gene or part thereof likely to carry the mutation into a plurality of fragments, such as by digestion of said relevant gene by selected restriction enzymes,
- hybridizing these fragments to complementary oligonucleotide probes, preferably a series of labelled probes recognizing under stringent conditions, all of the parts of the relevant gene of a corresponding control DNA of a strain non-resistant to the corresponding antibiotic,
- and relating the absence of hybridization of at least one of said oligonucleotide probes to any of the DNA fragments of the relevant gene of the mycobacterium under study as evidence of the presence of a mutation and, possibly, of a resistance to the corresponding antibiotic, particularly as compared to the runing of the test under the same conditions with the same oligonucleotides on the relevant gene(s) obtained from a strain (strains) not resistant to said antibiotic.

Another process alternative (SSCP analysis, i.e. analysis of Single Stranded Conformation Polymorphisms) comprises:

- digesting the DNA to be analyzed, particularly of the relevant gene,
- amplifying the fragments obtained, e.g. by PCR,
- recovering the amplified fragments, and
- separating them from one another according to sizes, e.g. by causing them to migrate, for instance on an electrophoretic gel,
- comparing the sizes of the different fragments with those

obtained from the DNA(s) of one or several control strains not resistant to the antibiotic, which had been subjected to a similar assay, and

- relating the polymorphism possibly detected to the existence of a mutation in the relevant gene, accordingly to a possible resistance to the corresponding antibiotic of the strain from which the DNA under study had been obtained.

Needless to say that any other method, including classical sequencing techniques, can resorted to for the achievement of the same purpose.

This method includes that known under the expression "oligotyping" for the detection of polymorphisms, reference is advantageously made to the method discloses by Orita et al. (reference was already made thereto herebefore) for the detection of polymorphisms based on the conformation of single strands.

The relevant gene in the case of resistance to isoniazid is of course the katG gene or a fragment thereof.

In the case of resistance to rifampicin, the relevant gene happens to be the rpoB gene which codes for the ßsub-unit of the RNA polymerases of said mycobacteria, or when only part of that gene is being used, preferably that part which includes the codons 400 to 450 of that rpoB gene.

Finally, in the case of resistance to steptomycin, the relevant gene contemplated is that of the rpsL gene that codes for the S12 protein of the small ribosome sub-unit or, when only part of said fragment is being used, preferably that part which includes the codon at the 43 position.

A preferred procedure, particularly in relation to the process alternative making use of PCR amplification is disclosed hereafter.

DNA is obtained from a biological sample (e.g. blood or

sputum) after removal of the cellular debris and lysis of the bacterial cells with an appropriate lysis buffer. PCR amplication can be carried out by classical methods, using a pair of primers, whose sequences are respectively complementary to fragments of each of the strands of the DNA to be amplified.

The procedure may be run further as follows:

- the amplification products (comprising e.g. from 100 to 300 nucleotides) are digested by means of suitable restriction endonuclease,
- the ADN strands obtained from the amplification medium are subjected to denaturation,
- the monostranded DNA strands are deposited on a neutral 5% polyacrylamid gel,
- the monostranded DNA strands are caused to migrate on said gel by means of electrophoresis,
- the DNA fragments that migrated on the polyacrilamid gel are transferred onto a nylon membrane according to a usual electrophoretic blotting technique and hybridized to labelled probes, for instance ^{32}P labelled probes, and
- the migration distances of the DNA fragments subjected to analysis are compared to those obtained from controls obtained under the same conditions of amplification, digestion, denaturation electrophoresis and transfer onto a nylon membrane, whereby said DNA had been obtained from an identical bacterial strain yet sensitive to the antibiotic under study.

For the production of the PCR primers as well as of the polygonucleotides probes used in the above disclosed "oligotyping" procedures, use is advantageously made of those complementary to the rpoB gene of wild M.tuberculosis inserted in a plasmid deposited under number I-12167 at the

CNCM on September 15, 1992.

The invention also relates more particularly to the nucleotidic sequence of a fragment of rpsL gene of Mycobacterium tuberculosis coding for the S12 protein of the small ribosome sub-unit, as well as to the nucleotidic sequence of a mutated rpsL gene fragment deemed responsible of the resistance to streptomycin.

By amplification of that nucleotidic sequence, the nucleotide sequence of the full rpsL gene can be obtained.

Further illustration of the invention will be provided in the following description of additional examples, having regard to the drawings in which:

- **figure 10** represents digrammatically the PCR strategy used for the study of different M.Leprae isolates, showing the coding sequence of rpoB sequence, whereby the sequenced regions are shown by hatched parts, and the position and reference of the amplification primers used being indicated on the upper line, whereas the sequencing primers are indicated bellow it;
- **figure 11** represents (A) the nucleotidic sequence of a short region of rpoB carrying the mutations that confer resistance to rifampicin with an indication of the changes of bases in the corresponding alleles and (B) a comparison between the aminoacids sequences of the domain I of region II of the β-sub-unit of the RNA polymerase of E.coli and M.Leprae, whereby the numbers of the residues and the differences in the mutated aminoacids have been indicated; the mutated aminoacid residues associated with rifampicin resistance as well as the frequency of its occurrences have been represented too,
- **figure 12** shows a complete sequence of the rpoB gene of M.Leprae,

- **figure 13** represents the sequence of part of the rpoB gene of M.tuberculosis,
- **figure 14** represents the sequence of a part of the rpsL gene of M.tuberculosis; both the sequence of the full rpsL gene of M.Leprae and that of its expression product, that is the S12 protein (whose starting aminoacid is noted by 1) are indicated. The positions of the ML51 and ML52 primers, as well as of the sequences of part of the rpsL gene of M.tuberculosis are provided belows those of M.Leprae. Only those positions which are different and the corresponding aminoacid changes are indicated.
- **figure 15** represents the wild DNA sequence of the rpsL gene fragment coding for the S12 protein of the small ribosome sub-unit that is responsible for the resistance to streptomycin, as well as the corresponding aminoacid sequence of the S12 protein.

Example relative to the detection of rifampicin resistance of

The sensitivity to rifampicin has been determined in mice as disclosed by Grosset et al. (and Int. J. Lepr. 57:607-614). The cells of M.Leprae were obtained from mouse paws according to classic procedures. All resistant strains were able to grow in mice which received daily doses of 20 mg/Kg of rifampicin, whereas sensitive strains were killed at low rifampicin concentrations, less than 2 mg/Kg.

Relevant regions of the rpoB gene of extracted DNA was initiated upon using two pairs of biotinylated primers, whose sequences appear in the following table.

TABLE

Primer	Séquence
Brpo22	CAGGACGTCGAGGCGATCAC
rpo23	AACGACGACGTGGCCAGCGT
Brpo24	CAGACGGTGTTTATGGGCGA
rpo25	TCGGAGAAACCGAAACGCTC
rpo32	TCCTCGTCAGCGGTCAAGTA
rpo33	CTTCCCTATGATGACTG
rpo34	GGTGATCTGCTCACTGG
rpo35	GCCGCAGACGCTGATCA
rpo36	TTGACCGCTGACGAGGA
rpo37	GCCAGCGTCGATGGCCG

Upon using conventional techniques, amplification products comprising 310 and 710 bp were respectively obtained as shown in figure 1. The localization of the sequences of the different primers used in the table is also indicated on figure 10.

The DNAs obtained have been sequenced on the basis of the rpoB sequence of isolates sensitive to rifampicin. A plasmid containing the sequence of that gene has been deposited at CNCM on September 15, 1992 under number I-1266. Biotinylated PCR products were concentrated from the PCR reaction mixtures by contacting with streptavidin coated beads under agitation. The biotinylated strands attached to the beads were then recovered and sequenced. The sequences obtained were compared to the sequence of the rpoB gene of a wild type stain. Significant results were obtained as a result of sequencing of the wild gene (of a mycobacterium sensitive to rifampicin) and of corresponding sequences of the ß-sub-unit of four mutant strains resistant to rifampicin (**figure 11**).

Results were obtained starting from 102 strands obtained from patients infected with M.tuberculosis. Among this 102 strands 53 were sensitive to rifampicin and 49 resistant to rifampicin. The mutation was localized in the region 400-450 in 43 of the mutants and among the latter, the mutation occured in the region of ^{425}Ser into leu.

Example of detection of the resistance of mycobacteria to streptomycin

The culture of M.tuberbulosis strains and the test of their sensitivity to streptomycin have been carried out by the method of proportions on a Löwenstein-Ierva medium (Laboratory Method for Clinical Mycobacteriology - Hugo David - Véronique Lévy Frébault, M.F. Thorel, published by Institut

Pasteur).

The nucleotide sequence of the rpsL gene of M.Leprae led, by sequence analogy, to the construction of two primers, ML51 (CCCACCATTCAGCAGCTGGT) et ML52 (GTCGAGCGAACCGCGAATGA) surounding regions including putative mutation sites liable of being responsible for the streptomycin resistance and suitable for the PCR reaction. The DNA of the used M.tuberculosis used as a matrix has enabled one to obtain a rpsL fragment of 306 pb. The nucleotide sequence of the sequenced fragments exhibited 28 differences with that of M.Leprae.

The rpsL genes or 43 strands of M.tuberculosis, among which 28 were resistant, have been amplified both by PCR and the SSCP technique.

DNA was extracted from 200 µl aliquots of M.tuberculosis samples (in average 10^4 to 10^5 bacteria) covered by 100µl of mineral oil by a congelation-decongelation technique (Woods and Cole, 1989 FEBS. Microbiol. Lett,65:305-308).

After electrophoresis of the DNA strands tested a mutation was shown in 16 of the mutants. In order to establish the nature of the mutation in the 16 strands under consideration, the corresponding rpsL gene fragments were amplified by PCR using the ML51 and the ML52 primers and their respective nucleotide sequences were determined.

The sequences obtained were compared to the sequence of the wild type rpsL gene. The single difference was found with the wild sequence ; codon 43, AAG, was mutated into AGG and, consequently, the lys-42 aminoacid was replaced by Arg.

The invention relates also to the "mutated" DNA fragments. They can in turn be used as hybridization probes for use for the dectection in suitable hybridization

procedures and for the detection of similar mutation in DNA extracted from a M.tuberbulosis strain suspected to include resistance to any one of the above illustrated antibiotics.

The invention further relates to kits for the resistance of mycobacteriae to isoniazid, rifampicin or analogues thereof, and streptomycin.

The invention further relates to a kit for the *in vitro* diagnostic of the resistance of a bacteria of a mycobacterium genus to isoniazid, characterized in that it comprises:

- means for carrying out for a genic amplification of the DNA of the katG gene or of a fragment thereof,
- means to bring into evidence one or several mutations on the amplification products so obtained,
- a preparation of control DNA of a katG gene of a strain of said bacteria sensitive to isoniazid or of a fragment thereof,
- optionally, a control preparation of a DNA of the katG gene of an isoniazid-resistant mycobacterium strain.

The invention further relates to a kit for the *in vitro* diagnostic of the resistance of a bacteria of a mycobacterium genus to rifampicin or its analogues, characterized in that it comprises:

- means for carrying out for a genic amplification of the DNA of the rpoB gene or of the ß-sub-unit of the RNA polymerase of said mycobacteria, or of a fragment thereof,
- means to bring into evidence one or several mutations on the amplification products so obtained,
- a preparation of control DNA of a rpoB gene coding for the ß-sub-unit of the RNA polymerase of a strain of said bacteria sensitive to rifampicin or of a fragment thereof,
- optionally, a control preparation of a DNA of the rpoB gene of an isoniazid-resistant mycobacterium strain.

Similarly, the invention pertains to a kit for the *in vitro* diagnostics of the resistance of the *M.tuberculosis* to streptomycin, characterized in that it includes:

- means for carrying out a genic amplification of the *rpsL* gene coding for the S12 protein of the small ribosome sub-unit, or fragment thereof,
- means which enable the bringing to evidence of one or several mutations on the amplification products obtained,
- a control preparation of a DNA sequence of the *rpsL* gene coding for the S12 protein of the small sub-unit of the ribosome of a *M.tuberculosis* strain sensitive to streptomycin, and
- optionally, a control preparation of a DNA sequence of a *rpsL* gene coding for the S12 protein of the small sub-unit of the ribosome of a strain of *M.tuberculosis* resis tant to streptomycin.

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CLAIMS

WHAT IS CLAIMED IS:

1. A process for the detection of a resistance to an antibiotic in a mycobacterium which comprises detecting a mutation in a gene selected from the group comprising the katG gene or fragment thereof, the rpoB gene or fragment thereof and the rpsL gene or fragment thereof.

2. A process of claim 1 for detecting in vitro the presence of nucleic acids of a Mycobacterium tuberculosis resistant to isoniazid, wherein the process comprises the steps of:

- contacting said nucleic acids previously made accessible to a probe if required under conditions permitting hybridization;
- detecting any probe that had hybridized to said nucleic acids;

wherein said probe comprises a nucleic acid sequence, which is 2.5 kb EcoRV-KpnI fragment of plasmid pYZ56 or of part thereof, and wherein said fragment contains a BamHI cleavage site, wherein said part is nonetheless sufficiently long to provide for the selectivity of the in vitro detection of a Mycobacterium tuberculosis resistant to isoniazid.

3. The process as claimed in claim 2, which comprises the steps of :

(A) depositing and fixing nucleic acids of Mycobacterium tuberculosis on a solid support, so as to make the nucleic acids accessible to a probe;

(B) contacting said fixed nucleic acids from step (A) with the probe under conditions permitting hybridization;

(C) washing said filter resulting from step (B), so as to eliminate any non-hybridized probe; and then

(D) detecting any hybridized probe on said washed filter resulting from step (C);

4. The process of claim 2 or 3 wherein said probe comprises a nucleic acid sequence which encodes a polypeptide of the formula APLNSWPDNASLDKAR-RLLWPSKKKYGKKLSWADLIV.

5. A process as claimed in any of claims 2 to 4, wherein the probe has a radioactive label selected from the group consisting of radioactive, enzymatic, fluorescent, and luminescent labels.

6. The use of the process of any one of claims 2 to 5 for the detection of the presence of Mycobacterium tuberculosis resistant to isoniazid in a bacteria-containing sample suspected of containing Mycobacterium tuberculosis resistant to isoniazid, whereby the detection of the probe that had hybridized, particularly in the form of a hybrid DNA complex that it either forms or had formed with DNA initially present in said sample, is indicative of the presence in said sample of Mycobacterium tuberculosis resistant to isoniazid.

7. The use of claim 6, wherein prior to the contacting of said DNA with said probe, said bacteria had been separated from said sample and immobilized on a DNA binding support, such as a nitrocellulose membrane.

8. A kit for the detection of Mycobacterium tuberculosis resistant to isoniazid, wherein the kit comprises:

(A) a container means containing a probe, preferably labelled by a label selected from the group consisting of radioactive, enzymatic, fluorescent, and luminescent labels, comprising a nucleic acid sequence, which is a 2.5 kb EcoRV-KpnI fragment of plasmid pYZ56 or part thereof, wherein said fragment contains a BamHI cleavage site and

nonetheless sufficiently long to provide for the selectivity of the in vitro detection of a Mycobacterium tuberculosis resistant to isoniazid; and

(B) a container means containing a control preparation of nucleic acid.

9. A nucleic acid probe for detecting Mycobacterium tuberculosis resistant to isoniazid, wherein said probe consists of a 2.5 kb EcoRV-KpnI fragment of plasmid pYZ56, wherein said fragment contains a BamHI cleavage site, or of a part of said fragment nonetheless sufficiently long to provide for the selectivity of the in vitro detection of a Mycobacterium tuberculosis resistant to isoniazid.

10. The probe as claimed in claim 9, which is DNA free of human serum proteins or human tissue or both, viral proteins, bacterial proteins, and nucleotide sequences encoding said proteins.

11. A hybrid duplex molecule consisting essentially of the probe of claim 9 hydrogen bonded to a nucleotide sequence of complementary base sequence.

12. A process for selecting a nucleotide sequence of a Mycobacterium tuberculosis resistant to isoniazid from a group of nucleotide sequences, comprising the step of determining which of said nucleotide sequences hybridizes to a probe as claimed in claim 9 or 10.

13. A process for selecting a compound active against Mycobacterium tuberculosis comprising the step of determining the reactivity of the compound on INH-resistant Mycobacterial strains.

14. A nucleotide sequence comprising the 350 base sequence or a portion thereof as described in Figure 2.

15. A process for detecting point mutations or partial

deletion of the KatG gene comprising contacting a sample of Mycobacterium tuberculosis with the probe of claim 9 or 10.

16. The process of claim 1 for the detection of resistance to the selected antibiotic which comprises:

- fragmenting the relevant gene or part thereof likely to carry the mutation into a plurality of fragments, such as by digestion of said relevant gene by selected restriction enzymes,
- hybridizing these fragments to complementary oligonucleotide probes, preferably a series of labelled probes recognizing under stringent conditions, all of the parts of the relevant gene of a corresponding control DNA of a strain non-resistant to the corresponding antibiotic,
- and relating the absence of hybridization of at least one of said oligonucleotide probes to any of the DNA fragments of the relevant gene of the mycobacterium under study as evidence of the presence of a mutation and, possibly, of a resistance to the corresponding antibiotic, particularly as compared to results obtained upon running the test under the same conditions with the same oligonucleotides on the relevant gene(s) obtained from a strain (strains) not resistant to said antibiotic, wherein said relevant gene is either the katG gene or a fragment thereof, the rboB gene or a fragment thereof, the rpsL gene or a fragment thereof.

17. The process of claim 1 which comprises:

- digesting the DNA to be analyzed, particularly of the relevant gene,
- amplifying the fragments obtained, e.g. by PCR,
- recovering the amplified fragments, and
- separating them from one another according to sizes, e.g. by causing them to migrate, for instance on an electrophoretic gel,

- comparing the sizes of the different fragments with those obtained from the DNA(s) of one or several control strains not resistant to the antibiotic, which had been subjected to a similar assay, and
- relating the polymorphism possibly detected to the existence of a mutation in the relevant gene, accordingly to a possible resistance to the corresponding antibiotic of the strain from which the DNA under study had been obtained, wherein said relevant gene is either the katG gene or a fragment thereof, the rboB gene or a fragment thereof, the rpsL gene or a fragment thereof.

18. A kit for the in vitro diagnostic of the resistance of a bacteria of a mycobacterium genus to isoniazid, characterized in that it comprises:
- means for carrying out for a genic amplification of the DNA of the katG gene or of a fragment thereof,
- means to bring into evidence one or several mutations on the amplification products so obtained,
- a preparation of control DNA of a katG gene of a strain of said bacteria sensitive to isoniazid or of a fragment thereof,
- optionally, a control preparation of a DNA of the katG gene of an isoniazid-resistant mycobacterium strain.

19. A kit for the in vitro diagnostic of the resistance of a bacteria of a mycobacterium genus to rifampicin or its analogues, characterized in that it comprises:
- means for carrying out for a genic amplification of the DNA of the rpoB gene or of the ß-sub-unit of the RNA polymerase of said mycobacteria, or of a fragment thereof,
- means to bring into evidence one or several mutations on the amplification products so obtained,
- a preparation of control DNA of a rpoB gene coding for the

ß-sub-unit of the RNA polymerase of a strain of said bacteria sensitive to rifampicin or of a fragment thereof,

- optionally, a control preparation of a DNA of the rpoB gene of an isoniazid-resistant mycobacterium strain.

20. A kit for the in vitro diagnostics of the resistance of the M.tuberculosis to streptomycin, characterized in that it includes:

- means for carrying out a genic amplification of the rpsL gene coding for the S12 protein of the small ribosome sub-unit, or fragment thereof,
- means which enable the bringing to evidence of one or several mutations on the amplification products obtained,
- a control preparation of a DNA sequence of the rpsL gene coding for the S12 protein of the small sub-unit of the ribosome of a M.tuberculosis strain sensitive to streptomycin, and
- optionally, a control preparation of a DNA sequence of a rpsL gene coding for the S12 protein of the small sub-unit of the ribosome of a strain of M.tuberculosis resistant to streptomycin.

FIG. 1

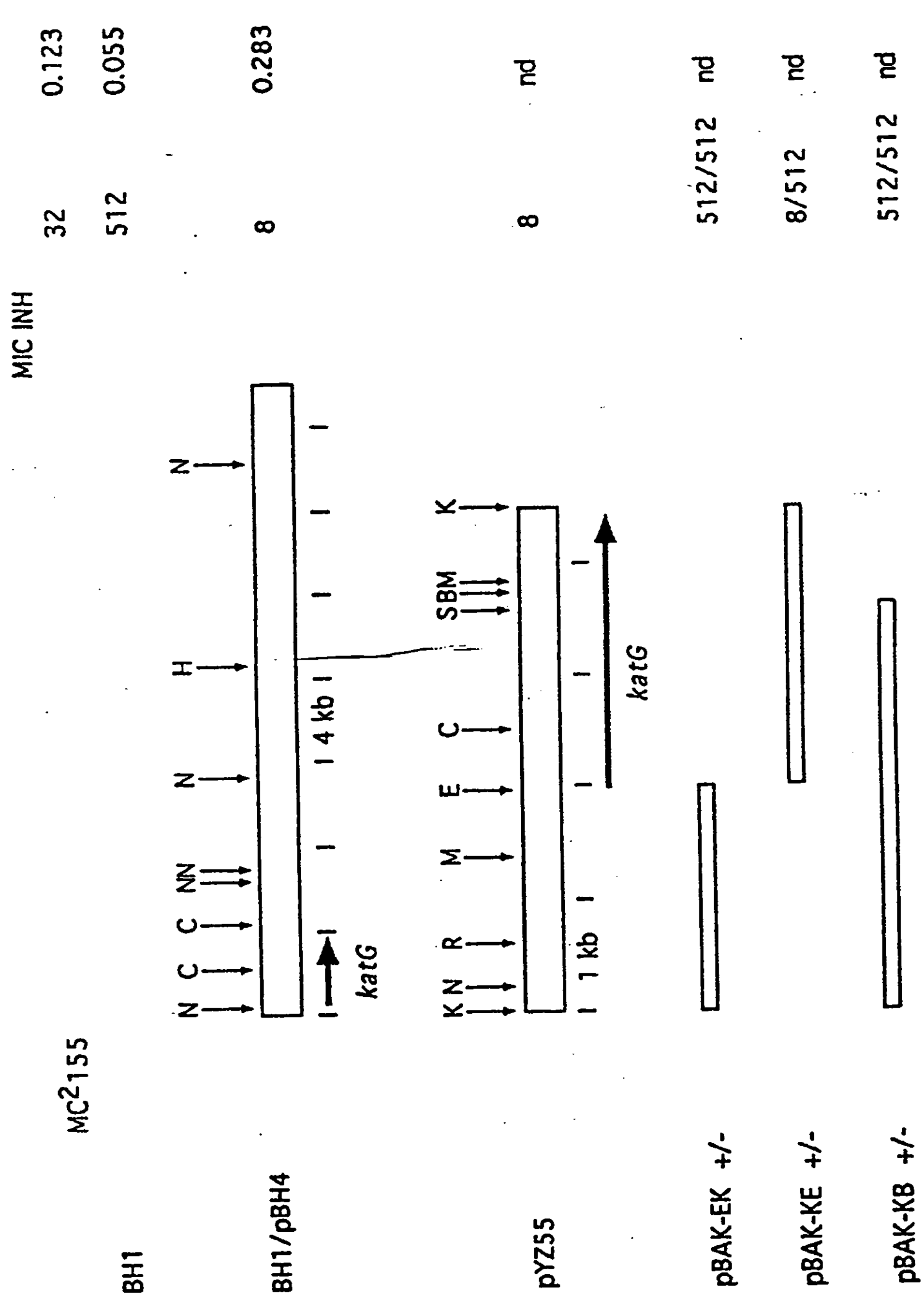

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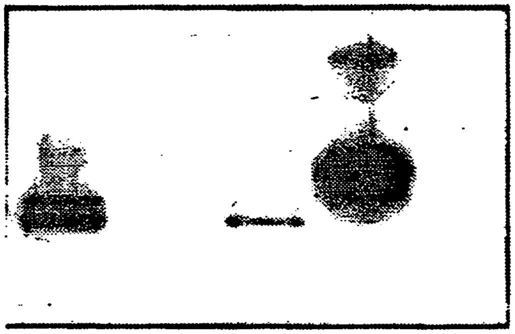
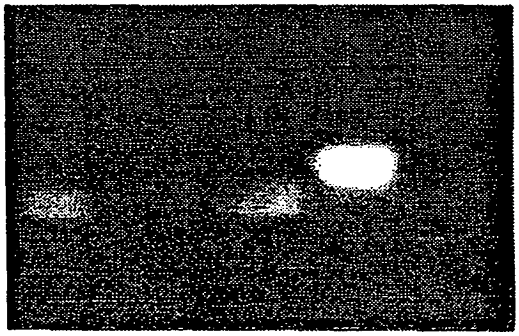
FIG. 2A



1 2 3 4 5

FIG. 2B



1 2 3 4 5

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FIG. 2C(1)

```
<------------------lacZ'---------------------------->

M T M I T P S L H A C R S T L E D P H P T L R
ATGACCATGATTACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCATCCGACACTTCGCG
         10        20        30        40        50        60        70

katG------>

M S T S D D - - I H N T T A T G K C P F H Q G
. :                   * * : : :     :
M P E Q H P P I T E T T T G A A S N G C P V V
GTGCCCGAGCAACACCCACCCATTACAGAAACCACCACCGGAGCCGCTAGCAACGGCTGTCCCGTCGTGG
       130       140       150       160       170       180       190

N Q L R V D L L N Q H S N R S N P L G E D F D
*   *   :   : *   *   :     : : * : *     * *
N R L N L K V L H Q N P A V A D P M G A A F D
AACCGGCTCAATCTGAAGGTACTGCACCAAAACCCGGCCGTCGCTGACCCGATGGGTGCGGCGTTCGACT
       250       260       270       280       290       300       310
```

FIG. 2C(2)

```
D  H  I  R  D  H  S  P  I  T  P  T  P  G  R  N  A
ATCACATCCGTGATCACAGCCCCGATAACACCAACTCCTGGAAGGAATGCT
      80        90        100       110       120

G  H  D  Q  S  A  G  A  G  T  T  T  R  D  W  W  P  E.coli
*  *           :        *  :  :        *  *  *  *
G  H  M  K  Y  P  V  E  G  G  G  N  Q  D  W  W  P  M.tub
GTCATATGAAATACCCCGTCGAGGGCGGCGGAAACCAGGACTGGTGGCCC
      200       210       220       230       240

Y  R  K  E  F  S  K  L  D  Y  Y  G  L  K  K  D  L  E.coli
*        *  :  :                 :  *     :  *  :
Y  A  A  E  V  A  T  S  R  L  D  A  L  T  R  D  I  M.tub
ATGCCGCGGAGGTCGCGACCAGTCGACTTGACGCCCTGACGCGGACATC
      320       330       340       350       360
```

FIG. 3

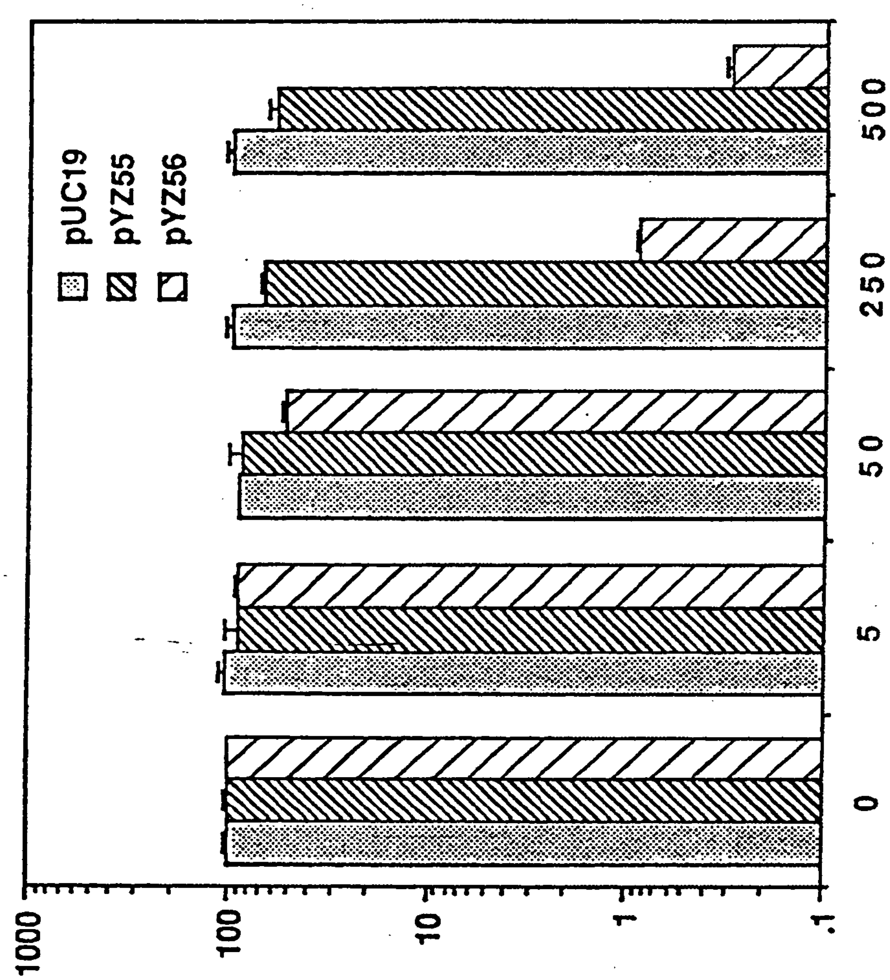


FIG. 4A

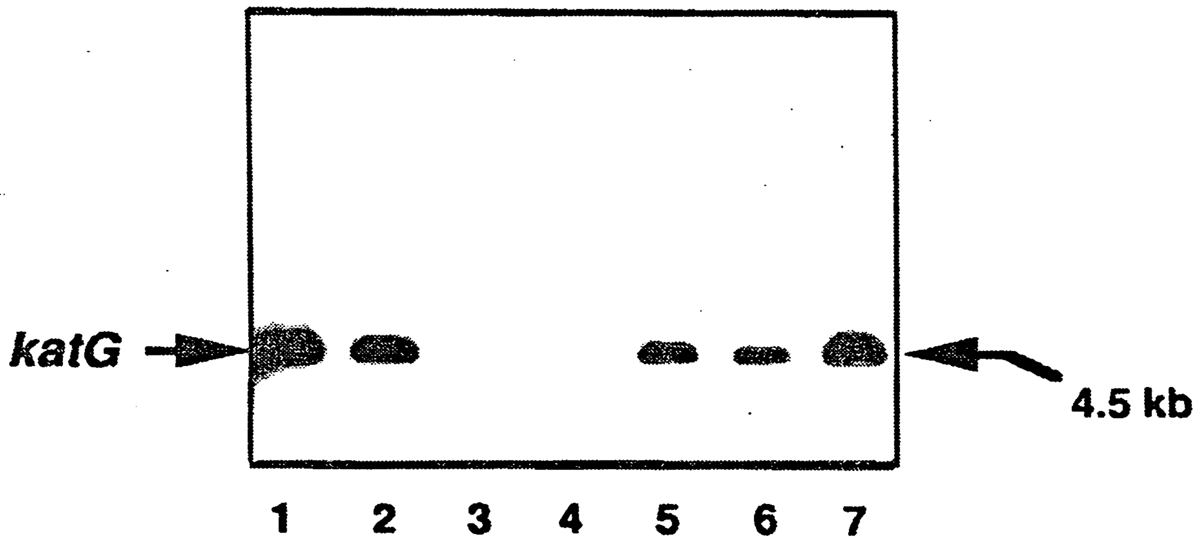
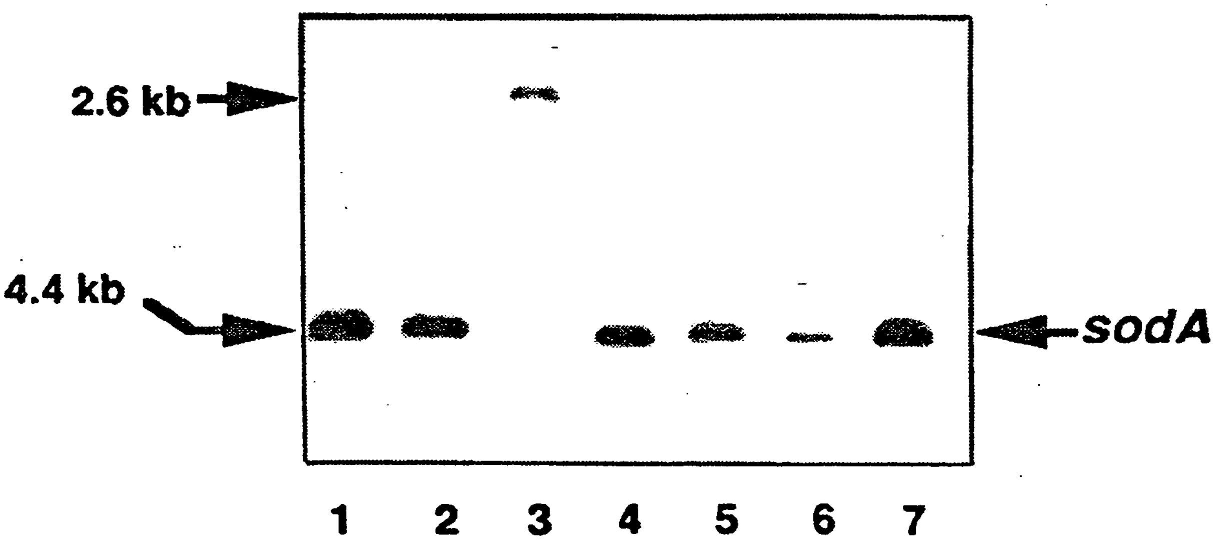


FIG. 4B



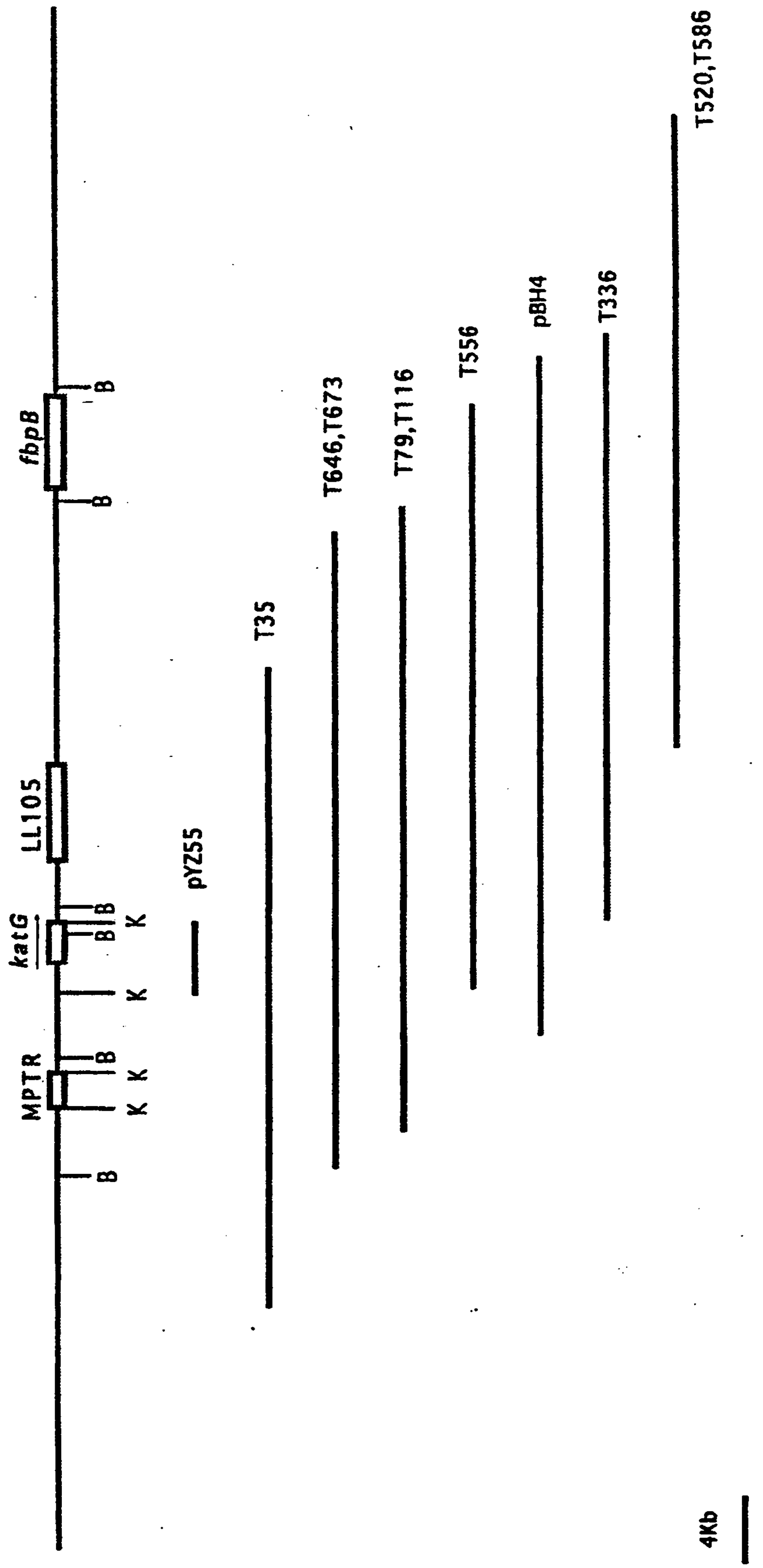

fbpB
B
LL105
katG
MPTR
K
pYZ55
T35
T646,T673
T79,T116
T556
pBH4
T336
T520,T586
4Kb

FIG. 5

FIGURE 6A(1)

```
GGTACCGTGA GGCGATGGGT GGCCCGGGGC CCGGCTGTCT GGTAAGCGCG GCCGCAAAAC     60
AGCTGTACTC TCGAATCCCA GTTAGTAACA ATGTGCTATG GAATCTCCAA TGACGAGCAC    120
ACTTCACCGA ACCCCATTAG CCACCGCGGG GCTGGCGCTC GTAGTGGCGC TGGGTGGCTG    180
CGGGGGCGGG GGCGGTGACA GTCGAGAGAC ACCGCCATAC GTGCCGAAAG CGACGACCGT    240
CGACGCAACA ACGCCGGCGC CGGCCGCCGA GCCACTGACG ATCGCCAGTC CCATGTTCGC    300
CGACGGCGCC CCGATCCCGG TGCAATTCAG CTGCAAGGGG GCCAACGTGG CCGCCACCGT    360
TGACGTGGTC GTCGCCCGCG GCGAGCGAAC TGGCACTCGT CGTCGATGAC CCCGACGCGG    420
TCGGCGGACT GTACGTGCAC TGGATCGTGA CCGGAATCGC CCCTGGCTCT GGCAGCACGG    480
CGGATGGTCA GACTCCTGCT GGTGGGCACA GCGTGCCGAA TTCTGGTGGT CGGCAAGGAT    540
ACTTCGGTCC ATGCCCGCCG GCGGGCACCG GGACACACCA CTACCGGTTT ACCCTCTACC    600
ACCTTCCTGT CGCGCTCCAG CTGCCACCGG GAGCCACGGG AGTCCAAGCG GCACAGGCGA    660
TAGCACAGGC CGCCAGCGAC AGGCCCGGCT CGTCGGCACA TTCGAAGGCT GACGCCGCGG    720
CATCCCTGGC GAGGTGGTCG AAACCCTGGC TTCTCCAATT GCGCCTGGCG ACAATGATCA    780
ATATGGAATC GACAGTGGCG CACGCATTTC ACCGGTTCGC ACTGGCCATC TTGGGGCTGG    840
CGCTCCCCGT GGCGCTAGTT GCCTACGGTG GCAACGGTGA CAGTCGAAAG GCGGCGGCCG    900
TGGCGCCGAA AGCAGCAGCG CTCGGTCGGA GTATGCCCGA AACGCCTACC GGCGATGTAC    960
TGACAATCAG CAGTCCGGCA TTCGCCGACG GTGCGCCGAT CCCGGAACAG TACACCTGCA   1020
AAGGAGCCAA TATCGCGGCC TCCGTTGACC TGGTCGGCGC CGTTTGGCGG CGCACTCGTT   1080
GTCGATGATC CGGACCACCT CGCGAACCTT ACGTCCATTG GATCGTGATC GGGATCGCCC   1140
CTGGTGCTGG CAGCAGCCGA TGGTGAGACT CCCGGTGGCG GAATCAGCCT GCCGAACTCC   1200
AGCGGTCAGC CCGCATACAC CGGCCCCTGC CCGCCGGCGG GCACCGGGAC ACACCACTAC   1260
```

FIGURE 6A(2)

```
CGGTTTACCC TCTACCACCT TCCTGCCGTG CCTCCACTCG CGGGACTGGC TGGGACACAA 1320
GCGGCGCGGG TGATCGCGCA GGCCGCCACC ATGCAGGCCC GGCTCATCGG AACATACGAA 1380
GGCTGATCCA CCCGCCATCC CACGATCCAG CGGCCCCGGG CGATCGGGTC CTAGCAGACG 1440
CCTGTCACGC TAGCCAAAGT CTTGACTGAT TCCAGAAAAG GGAGTCATAT TGTCTAGTGT 1500
GTCCTCTATA CCGGACTACG CCGAACAGCT CCGGACGGCC GACCTGCGCG TGACCCGACC 1560
GCGCGTCGCC GTCCTGGAAG CAGTGAATGC GCATCCACAC GCCGACACGG AAACGATTTT 1620
CGGTGCCGTG CGTTTTGCGC TGCCCGACGT ATCCGGCAAG CCGTGTACGA CGTGCTGCAT 1680
GCCCTGACCG CCGCGGGCTT GGTGCGAAAG ATCCAACCCT CGGGCTCCGT CGCGCGCTAC 1740
GAGTCCAGGG TCGGCGACAA CCACCATCAC ATCGTCTGCC GGTCTTGCGG GGTTATCGCC 1800
GATGTCGACT GTGCTGTTGG CGAGGCACCC TGTCTGACGG CCTCGGACCA TAACGGCTTC 1860
CTGTTGGACG AGGCGGAGGT CATCTACTGG GGTCTATGTC CTGATTGTTC GATATCCGAC 1920
ACTTCGCGAT CACATCCGTG ATCACAGCCC GATAACACCA ACTCCTGGAA GGAATGCTGT 1980
GCCCGAGCAA CACCCACCCA TTACAGAAAC CACCACCGGA GCCGCTAGCA ACGGCTGTCC 2040
CGTCGTGGGT CATATGAAAT ACCCCGTCGA GGGCGGCGGA AACCAGGACT GGTGGCCCAA 2100
CCGGCTCAAT CTGAAGGTAC TGCACCAAAA CCCGGCCGTC GCTGACCCGA TGGGTGCGGC 2160
GTTCGACTAT GCCGCGGAGG TCGCGACCAG TCGACTTGAC GCCCTGACGC GGGACATCGA 2220
GGAAGTGATG ACCACCTCGC AGCCGTGGTG GCCCGCCGAC TACGGCCACT ACGGGCCGCT 2280
GTTTATCCGG ATGGCGTGGC ACGCTGCCGG CACCTACCGC ATCCACGACG GCCGCGGCGG 2340
CGCCGGGGGC GGCATGCAGC GGTTCGCGCC GCTTAACAGC TGGCCCGACA ACGCCAGCTT 2400
GGACAAGGCG CGCCGGCTGC TGTGGCCGGT CAAGAAGAAG TACGGCAAGA AGCTCTCATG 2460
GGCGGACCTG ATTGTTTTCG CCGGCAACCG CTGCGCTCGG AATCGATGGG CTTCAAGACG 2520
TTCGGGTTCG GCTTCGGGCG TCGACCAGTG GGAGACCGAT GAGGTCTATT GGGGCAAGGA 2580
```

FIGURE 6A(3)

```
AGCCACCTGG CTCGGCGATG ACGGTTACAG CGTAAGCGAT CTGGAGAACC CGCTGGCCGC  2640
GGTGCAGATG GGGCTGATCT ACGTGAACCC GGAGGCGCCG AACGGCAACC CGGACCCCAT  2700
GGCCGCGGCG GTCGACATTC GCGAGACGTT TCGGCGCATG GCCATGAACG ACGTCGAAAC  2760
AGCGGCGCTG ATCGTCGGCG GTCACACTTT CGGTAAGACC CATGGCGCCG GCCCGGCCGA  2820
TCTGGTCGGC CCCGAACCCG AGGCTGCTCC GCTGGAGCAG ATGGGCTTGG GCTGGAAGAG  2880
CTCGTATGGC ACCGGAACCG GTAAGGACGC GATCACCAGC GGCATCGAGG TCGTATGGAC  2940
GAACACCCCG ACGAAATGGG ACAACAGTTT CCTCGAGATC CTGTACGGCT ACGAGTGGGA  3000
GCTGACGAAG AGCCCTGCTG GCGCTTGGCA ATACACCGCC AAGGACGGCG CCGGTGCCGG  3060
CACCATCCCG GACCCGTTCG GCGGGCCAGG GCGCTCCCCG ACGATGCTGG CCACTGACCT  3120
CTCGCTGCGG GTGGATCCGA TCTATGAGCG GATCACGCGT CGCTGGCTGG AACACCCCGA  3180
GGAATTGGCC GACGAGTTCC GCAAGGCCTG GTACAAGCTG ATCCACCGAG ACATGGGTCC  3240
CGTTGCGAGA TACCTTGGGC CGCTGGTCCC CAAGCAGACC CTGCTGTGGC AGGATCCGGT  3300
CCCTGCGGTC AGCACGACCT CGTCGGCGAA GCAGATTGCC AGCCTTAAGA GCCAGATCCG  3360
GGCATCGGGA TTGACTGTCT CACAGCTAGT TTCGACCGCA TGGGCGGCGG CGTCGTCGTT  3420
CCGTGGTAGC GACAAGCGCG GCGGCGCCAA CGGTGGTCGC ATCCGCCTGC AGCCACAAGT  3480
CGGGTGGGAG GTCAACGACC CCGACGGATC TGCGCAAGGT CATTCGCACC CTGAAGAGAT  3540
CCAGGAGTCA TTCACTCGGC GCGGGAACAT CAAAGTGTCC TTCGCCGACC TCGTCGTGCT  3600
CGGTGGCTGT GCGCCACTAG AGAAAGCAGC AAAGGCGGCT GGCCACAACA TCACGGTGCC  3660
CTTCACCCCG GGCCCGCACG ATGCGTCGCA GGAACAAACC GACGTGGAAT CCTTTGCCGT  3720
GCTGGAGCCC AAGGCAGATG GCTTCCGAAA CTACCTCGGA AAGGGCAACC GTTGCCGGCC  3780
GAGTACATCG CTGCTCGACA AGGCGAACCT GCTTACGCTC AGTGCCCCTG AGATGACGGT  3840
GCTGGTAGGT GGCCTGCGCG TCCTCGGCGC AAACTACAAG CGCTTACCGC TGGGCGTGTT  3900
```

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FIGURE 6A(4)

```
CACCGAGGCC TCCGAGTCAC TGACCAACGA CTTCTTCGTG AACCTGCTCG ACATGGGTAT   3960
CACCTGGGAG CCCTCGCCAG CAGATGACGG GACCTACCAG GGCAAGGATG GCAGTGGCAA   4020
GGTGAAGTGG ACCGGCAGCC GCGTGGACCT GGTCTTCGGG TCCAACTCGG AGTTGCGGGC   4080
GCTTGTCGAG GTCTATGCGC CGATGACGCG GCAGGCGAAG TTCGTGACAG GATTCGTCGC   4140
TGCGTGGGAC AAGGTGATGA ACCTCGACAG GTTCGACGTG CGCTGATTCG GGTTGATCGG   4200
CCCTGCCCGC CGATCAACCA CAACCCGCCG CAGCACCCCG CGAGCTGACC GGCTCGCGGG   4260
GTGCTGGTGT TTGCCCGGCG CGATTTGTCA GACCCCGCGT GCATGGTGGT CGCACGGACG   4320
CACGAGACGG GGATGACGAG ACGGGGATGA GGAGAAAGGG CGCCGAAATG TGCTGGATGT   4380
GCGATCACCC GGAAGCCACC GCCGAGGAGT ACCTCGACGA GGTGTACGGG ATAATGCTCA   4440
TGCATGGCTG GGCGGTACAG CACGTGGAGT GCGAGCGACG GCCATTTGCC TACACGGTTG   4500
GTCTAACCCG GCGCGGCTTG CCCGAACTGG TGGTGACTGG CCTCTCGCCA CGACGTGGGC   4560
AGCGGTTGTT GAACATGCCG TCGAGGGCTC TGGTCGGTGA CTTGCTGACT CCCGGTATGT   4620
AGACCACCCT CAAAGCCGGC CCTCTTGTCG AAACGGTCCA GGCTACACAT CCGGACGCGC   4680
ATTTGTATTG TGCGATCGCC ATCTTTGCGC ACAAGGTGAC GGCCTTGCAG TTGGTGTGGG   4740
CCGACCGCGT GGTCGCTGGC CGTGGGCGGC GGACTTCGAC GAAGGTCGCG GTACC        4795
```

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FIGURE 6B(1)

```
  --T----T-- --CGAAGGCT GACGCCG--- --CGGCATCC CTGGCGAG-G TGGTCG-AAA
     *    *     *** ** * * * ***      **** *   ****  ** *  ** ** ***
  GGTACCGTGA GGCGATGGGT GGC-CCGGGG CCCGGC-TGT CTGGTAAGCG CGGCCGCAAA
  1          11         21         31         41         51
761        771        781        791        801        811
  CC--CTGG-C TTCTCCAATT GCGCCTG--- GCGACAATGA T-CAATATGG AATCGACAGT
   *  ***  * * *** ***   * ** *    *  ****** * *  ***** ****   ** *
  ACAGCTGTAC T-CTCGAAT- -C-CCAGTTA GTAACAATG- TGC--TATGG AATCTCCAAT
 61         71         81         91         101        111
821        831        841        851        861        871
  GGCG--CACG CATTTCACCG GTTCGCACTG GCCATCTTGG GGCTGGCGCT CCCCGTGGCG
  * **  ***   * *******    * ** *  **** *  ** ********** *   ******
  GACGAGCAC- -ACTTCACCG AACCCCATTA GCCACCGCGG GGCTGGCGCT CGTAGTGGCG
121        131        141        151        161        171
881        891        901        911        921        931
  CTAGTTGCCT ACGGTGGCAA ---CGGTGAC AGTCGAAAGG CGGCGGC--- CGTGGCGCCG
  ** * ** **  *** ***      ******* ****** **  *  ** *   ***    ****
  CTGGGTGGCT GCGGGGGCGG GGGCGGTGAC AGTCGAGAGA CACCGCCATA CGT---GCCG
181        191        201        211        221        231
941        951        961        971        981        991
  AAAGCAGCAG CG-CTCGGTC G--G--AGTA TGCCCGAAAC GCCTACCGGC GATGT-ACTG
  ***** * *  ** * ** ** *  *  *  *  *** *    * ***  *** * ** *  ****
  AAAGC-G-A- CGAC-CG-TC GACGCAACAA CGCCGG---C GCCGGCCGCC GA-GCCACTG
241        251        261        271        281        291
1001       1011       1021       1031       1041       1051
  ACAATCAGC- AGTCCGGCAT --TCGCCGAC GGTGCGCCGA TCCCGGAACA GTACACCTGC
  ** *** **  *****  ***   ******** ** ** **** ******  **   * ** ****
  ACGATC-GCC AGTCC--CAT GTTCGCCGAC GGCGCCCCGA TCCCGGTGCA ATTCAGCTGC
301        311        321        331        341        351
1061       1071       1081       1091       1101       1111
  AAAGGAGCCA ATATCGCGGC CTCCGTTGAC CTGGTCGGCG CC-GTTTGGC G-GCG-----
  ** ** **** *   * ** ** * ********  ****** ** ** *   *** * ***
  AAGGGGGCCA ACGTGGCCGC CACCGTTGAC GTGGTCGTCG CCCGC--GGC GAGCGAACTG
361        371        381        391        401        411
1121       1131       1141       1151       1161       1171
  -CACTCGTTG TCGATGATCC GGAC-CACCT CG-CGAACCT -TACGTCCAT TGGATCGTGA
   ******* * ******* **  *** *    * ** ** ** *  ***** **  **********
  GCACTCGTCG TCGATGACCC CGACGCGG-T CGGCGGAC-T GTACGTGCAC TGGATCGTGA
421        431        441        451        461        471
1181       1191       1201       1211       1221       1231
  TCGGGATCGC CCCTGG-TGC TGGCAGCA-- GCCGATGGTG AGACTCCCGG TGGCGGA-AT
   *** ***** ****** * * ********    ** ******  ******* *  *** **  *
  CCGGAATCGC CCCTGGCT-C TGGCAGCACG GCGGATGGTC AGACTCCTGC TGGTGGGCA-
481        491        501        511        521        531
```

FIGURE 6B(2)

```
1241      1251       1261       1271       1281       1291
    CAGCCTGCCG AACTCCAGCG GTCAGCCCGC ATACACCGGC CCCTGCCCGC CGGCGGGCAC
    **** ***** ** **   * * *** **   *   ****  ***   ** ******* **********
    CAGCGTGCCG AATTCTGGTG GTCGGCAAGG ATACTTCGGT CCATGCCCGC CGGCGGGCAC
541       551        561        571        581        591
1301      1311       1321       1331       1341       1351
    CGGGACACAC CACTACCGGT TTACCCTCTA CCACCTTCCT GCCGTGCCTC CA-CTCGC--
    ********** ********** ********** ********** * ** ** ** ** ** **
    CGGGACACAC CACTACCGGT TTACCCTCTA CCACCTTCCT GTCGCGC-TC CAGCT-GCCA
601       611        621        631        641        651
1361      1371       1381       1391       1401       1411
    --GGGACTGG CT--GGGA-- CACAAGCGGC GCGGGTGATC GCGCAGGCCG CCACCATG-C
      ****    * *   ****   * ********  * ** ***  ** ******* *** *  * *
    CCGGGA---G CCACGGGAGT C-CAAGCGGC ACAGGCGATA GCACAGGCCG CCAGC--GAC
661       671        681        691        701        711
1421      1431       1441       1451       1461
    AGGCCCGGCT CATCGGAACA TACGAAGGCT GATCCACCCG CCATCC
    ********** * **** ***
    AGGCCCGGCT CGTCGGCACA ---------- ---------- ------
721       731        741        751        761
```

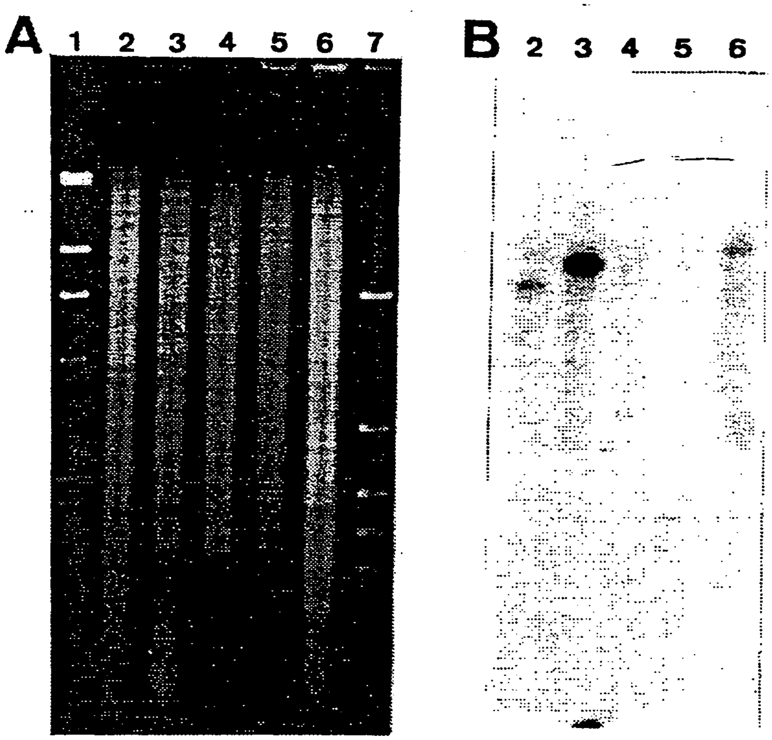

FIG. 7

FIGURE 8(1)

```
             1                                                                             70
(MTKATG)     MPEQHPPITE TTTGAASNGC PVVGHMKYPV EGGGNQDWWP NRLNLKVLHQ NPAVADPMGA AFDYAAEVAT
(ECKATG)     ..MSTSDDIH NTTATGKCPF HQGGHDQSAG AGTTTRDWWP NQLRVDLLNQ HSNRSNPLGE DFDYRKEF..
(STKATG)     ..MSTTDDTH NTLSTGKCPF HQGGHDRSAG AGTASRDWWP NQLRVDLLNQ HSNRSNPLGE DFDYRKEF..
(BSPERA)     ......MENQ NRQNAAQCPF HESVTNQSS. NRTTNKDWWP NQLNLSILHQ HDRKTNPHDE EFNYAEEFQ.
(CCP)        .......... .......... .......... .......... ...TTPLVHV ASVEKGRSYE DFQ.......
CONSENSUS    --MST-DDTH NTT---KCPF HQGGHDQSAG AGTTNRDWWP NQL--DLLHQ HSNRSNPLGE DFDY-KEF--

             71                                                                           140
(MTKATG)     SRLD...ALT RDIEEVMTTS QPWWPADYGH YGPLFIRMAW HAAGTYRIHD GRGGAGGGMQ RFAPLNSWPD
(ECKATG)     SKLDYY.GLK KDLKALLTES QPWWPADWGS YAGLFIRMAW HGAGTYRSID GRGGAGRGQQ RFAPLNSWPD
(STKATG)     SKLDYYSALK GDLKALLTDS QPWWPADWGS YVGLFIRMAW HGAGTYRSID GRGGAGRGQQ RFAPLNSWPD
(BSPERA)     .KLDYW.ALK EDLRKLMTES QDWWPADYGH YGPLFIRMAW HSAGTYRIGD GRGGASTGTQ RFAPLNSWPD
(CCP)        .KVYNAIALK ......LRED DEY..DNYIG YGPVLVRLAW HISGTWDKHD NTGGSYGGTY RFKKEFNDPS
CONSENSUS    SKLDYY-ALK -DLKALLTES QPWWPADYG- YGPLFIRMAW HGAGTYR--D GRGGAG-G-Q RFAPLNSWPD
                                              R   W H(108)

             141                                                                          210
(MTKATG)     NASLDKARRL LWPVKKKYGK KLSWADLIVF AGNRCARNRW ASRRSGSASG ...VDQWETD .EVYWGKEAT
(ECKATG)     NVSLDKARRL LWPIKQKYGQ KISWADLFIL AGNVALENSG FRTFGFGAGR ...EDVWEPD LDVNWGDEKA
(STKATG)     TVSLDKARRL LWPIKQKYGQ KISWADLFIL AGNVALENSG FRTFGFGAGR ...EDVWEPD LDVNWGDEKA
(BSPERA)     NANLDKARRC YGRSKRNTGT K.SLGPICSF WRAMSLLNRW VEKRLDSAAG PLTSGIRKKT FIGDRKKSGS
(CCP)        NAGLQNGFKF LEPIHKEFP. WISSGDLFSL GGVTAVQEMQ GPKIPWRCGR VDTPEDTTPD ........NG
CONSENSUS    NASLDKARRL LWPIK-KYGQ KISWADLFIL AGNVALEN-- FR--GF-AGR --TEDVWEPD LDVNWG-EKA
             N(138)

             211                                                                          280
(MTKATG)     WLGDDGYSVS DLENPLAAVQ MGLIYVNPEA PNGNPDPMAA AVDIRETFRR MAMNDVETAA LIVGGHTFGK
(ECKATG)     WLTHR.HPEA LAKAPLGATE MGLIYVNPEG PDHSGEPLSA AAAIRATFGN MGMNDEETVA LIAGGHTLGK
(STKATG)     WLTHR.HPEA LAKAPLGATE MDLIYVTPEG PNHSGEPLSA AAAIRATFGN MGMNDEETVA LIAGGHTLGK
(BSPERA)     PLNAIPVIAS SKTRSPRANG VNLRQPRRAG RQAGSKSRGI SA...ETFRR MGMNDEETVA LIAGGHTFGK
(CCP)        RL........ .......... .......... ....PDADKD AGYVRTFFQR LNMNDREVVA LM.GAHALGK
CONSENSUS    WLTHR-HPE- LAKAPLGATE MGLIYVNPEG PNHS--PLSA AAAIR-TF-R MGMNDEETVA LIAGGHTLGK
                                                                                    H(269)

             281                                                                          350
(MTKATG)     THGAGPADLV GPEPEAAPLE QMGLGWKSSY GTGTGKDAIT SGIEVVWTNT PTKWDNSFLE ILYGYEWELT
(ECKATG)     THGAGPTSNV GPDPEAAPIE EQGLGWASTY GSGVGADAIT SGLEVVWTQT PTQWSNYFFE NLFKYEWVQT
(STKATG)     THGPAAASHV GADPEAAPIE AQGLGWASSY GSGVGADAIT SGLEVVWTQT PTQWSNYFFE NLFKYEWVQT
(BSPERA)     AHRGGPATHV GPEPEAAPIE AQGLGWISSY GKGKGSDTIT SGIEGAWTPT PTQWDTSYFD MLFGYDWWLT
(CCP)        TH........ .......... .......... .......LKN SGYEGPWGAA NNVFTNEFYL NLLNEDWKLE
CONSENSUS    THGAGPASHV GP-PEAAPIE AQGLGWASSY GSGVGADAIT SG-EVVWTQT PTQW-N-FFE NLF-YEWVLT
             TH(275)                                    W(320)

             351                                                                          420
(MTKATG)     KSPAGAWQYT AKDGAGAGTI PDPFGGPGR. ..SPTMLATD LSLRVDPIYE RITRRWLEHP EELADEFRKA
(ECKATG)     RSPAGAIQFE AVD..APEII PDPFDPSKKR ..KPTMLVTD LTLRFDPEFE KISRRFLNDP QAFNEAFARA
(STKATG)     RSPAGAIQFE AVD..APDII PDPFDPSKKR XXKPTMLVTD LTLRFDPEFE KISRRFLNDP QAFNEAFARA
(BSPERA)     KSPAGAWQWM AVDPDEKDLA PDAEDPSKK. .VPTMMMTTD LALRFDPEYE KIARRFHQNP EEFAEAFARA
(CCP)        KNDANNEQWD SKSGY..... .......... ....MMLPTD YSLIQDPKYL SIVKEYANDQ DKFFKDFSKA
CONSENSUS    KSPAGA-Q-E AVDG-APDII PDPFDPSKKR --KPTMLVTD L-LRFDPEYE KISRRFLNDP E-F-EAFARA
                                                       D(380)
```

FIGURE 8(2)

```
          421                                                                        490
(MTKATG)  WYKLIHRDM. ...GPVARYL GPLVPKQTLL WQDPVPAVST TSSAKQIASL KSQIRASGLT VSQLVSTAWA
(ECKATG)  WFKLTHRDM. ...GPKSRYI GPEVPKEDLI WQDPLPQPIY NPTEQDIIDL KFAIADSGLS VSELVSVAWA
(STKATG)  WFKLTHRDM. ...GPKARYI GPEVPKEDLI WQDPLPQPLY QPTQEDIINL KAAIAASGLS ISEMVSVAWA
(BSPERA)  WFKLTHRDM. ...GPKTRYL GPEVPKEDFI WQDPIPEVDY ELTEAEIEEI KAKILNSGLT VSELVKTAWA
(CCP)     FEKLLENGIT FPKDAPSPFI FKTLEEQGL. .......... .......... .......... ..........
CONSENSUS WFKLTHRDM- ---GPK-RYI GPEVPKEDLI WQDP-PQ--Y -PTE-DII-L KAAIAASGL- VSELVS-AWA

          491                                                                        560
(MTKATG)  AASSFRGSDK RGGA.NGGRI RLQPQVGWEV NDPDGSAQGH SHPEEIQESF TRRGNIKVSF ADLVVLGGCA
(ECKATG)  SASTFRGGDK RGGA.NGARL ALMPQRDWDV N..AAAVRAL PVLEKIQ... ..KESGKASL ADIIVLAGVV
(STKATG)  SASTFRGGDK RGGA.NGARL ALAPQRDWDV N..AVAARVL PVLEEIQ... ..KTTNKASL ADIIVLAGVV
(BSPERA)  SAA..RSATR ISAATNGRRI RLAPQKDWEV NEPERLAKVL SVLRGHPA.. ..RTAEKSKH RRLDRLGGTL
(CCP)     .......... .......... .......... .......... .......... .......... ..........
CONSENSUS SASTFRGGDK RGGA-NGAR- -LAPQRDW-V N-P--AARVL -VLEEIQ--- ---T--KASL AD-IVL-GVV

          561                                                                        630
(MTKATG)  PLEKAAKAAG HNITVPF... TPGPHDASQE QTDVESFAVL EPKADGFRN. ...YLGKGNR CRPSTSLLDK
(ECKATG)  GVEKAASAAG LSIHVPF... APGRVDARQD QTDIEMFELL EPIADGFRN. ...YRARLDV STTESLLIDK
(STKATG)  GIEQAAAAAR VSIHVPF... PPGRVDARHD QTDIEMFSLL EPIADGFRN. ...YRARLDV STTESLLIDK
(BSPERA)  RWKRQPATPA LMSKCHFSLA AAMRHKSKPM SKALPCWNRS QMASATIKSK STRFRRKSCS STKPSSSADR
(CCP)     .......... .......... .......... .......... .......... .......... ..........
CONSENSUS G-EKAAAAAG LSIHVPF--- APGR-DARQD QTDIEMF-LL EPIADGFRN- ---YRA-LDV STTES-LIDK

          631                                                                        700
(MTKATG)  ANLLTLSAPE MTVLVGGLRV LGANYKRLPL GVFTEASESL TNDFFVNLLD MGITWEPSPA DDGTYQGKD.
(ECKATG)  AQQLTLTAPE MTALVGGMRV LGGNFDGSKN GVFTDRVGVL SNDFFVNLLD MRYEWKATDE SKELFEGRDR
(STKATG)  AQQLTLTAPE MTVLVGGMRV LGTNFDGSQN GVFTDKPGVL STDFFANLLD MRYEWKPTDD ANELFEGRDR
(BSPERA)  PRNDGLSWR. .......FAR VGPNYRHLPH GVFTDRIGVL TNDFFVNLLD MNYEWVPTDS ..GIYEIRDR
(CCP)     .......... .......... .......... .......... .......... .......... ..........
CONSENSUS AQQLTL-APE MTVLVGGMRV LG-N-DG-PN GVFTDR-GVL -NDFFVNLLD MRYEWKPTD- ---L-EGRDR

          701                                                                     767
(MTKATG)  GSGKVKWTGS RVDLVFGSNS ELRALVEVYA PMTRQAKFVT GFVAAWDKVM NLDRFDVR.. .......
(ECKATG)  ETGEVKFTAS RADLVFGSNS VLRAVAEVYA SSDAHEKFVK DFVAAWVKVM NLDRFDLL.. .......
(STKATG)  LTGEVKYTAT RADLVFGSNS VLRALAEVYA CSDAHEKFVK DFVAAWVKVM NLDRFDLQ.. .......
(BSPERA)  KTGEVRWTAT RVDLIFGSNS ILRSYAEFYA QDDNQEKFVR DFINAWVKVM NADRFDLVKK ARESVTA
(CCP)     .......... .......... .......... .......... .......... .......... .......
CONSENSUS -TGEVKWTA- R-DLVFGSNS VLRALAEVYA -SDA-EKFVK DFVAAWVKVM NLDRFDL--- -------
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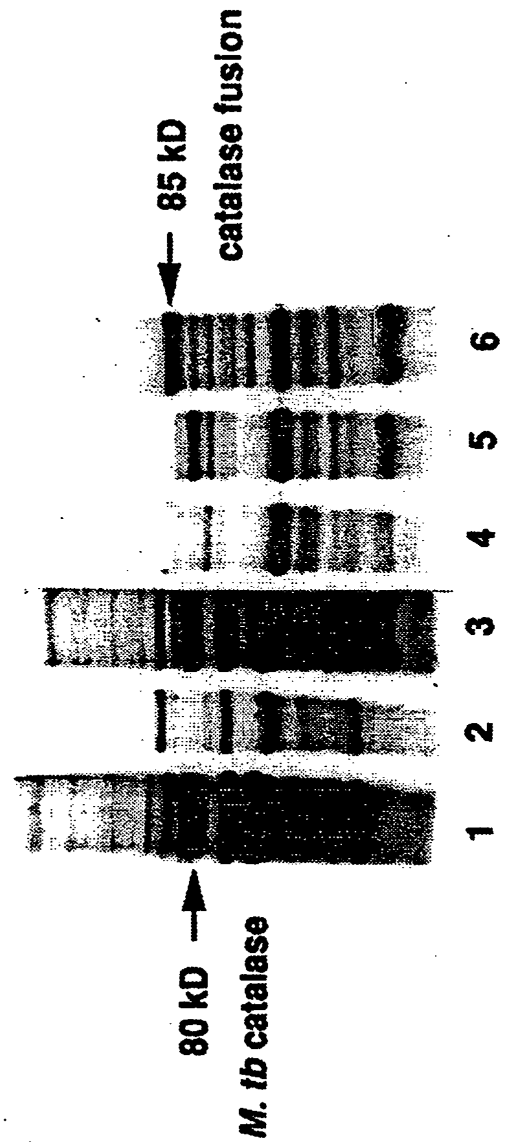
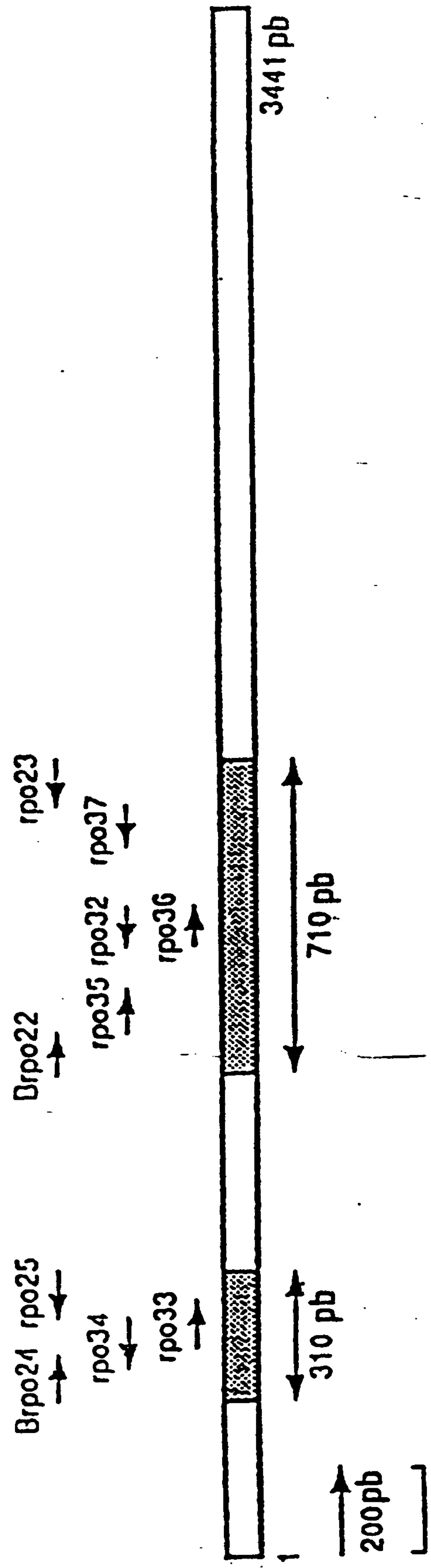


FIG. 9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rpo23
rpo37
rpo32
rpo36
rpo35
Brpo22
710 pb
rpo25
Brpo24
rpo34
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310 pb
200pb

FIG. 10

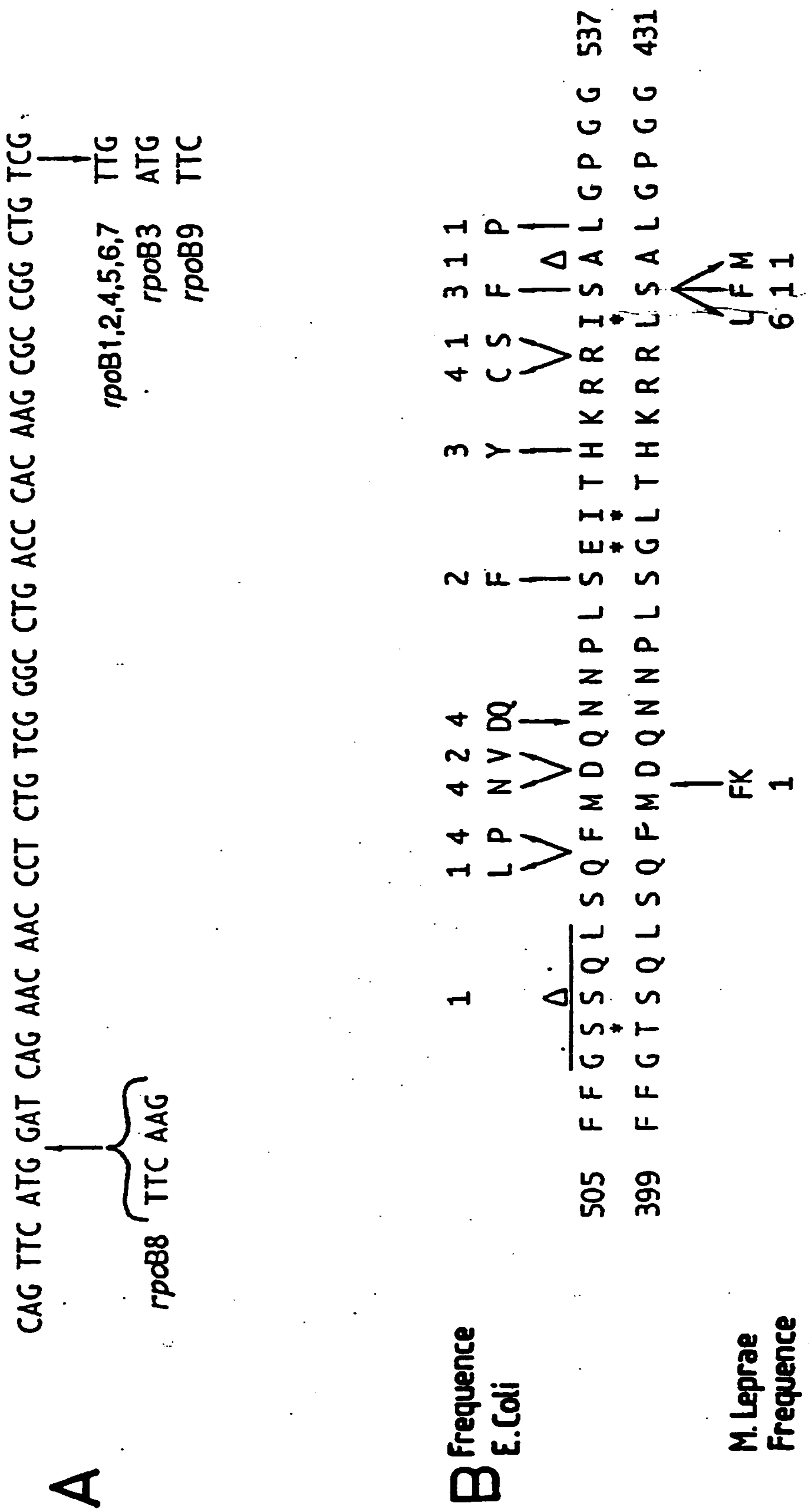
A
CAG TTC ATG GAT CAG AAC AAC CCT CTG TCG GGC CTG ACC CAC AAG CGC CGG CTG TCG
rpoB8 TTC AAG
rpoB1,2,4,5,6,7 TTG
rpoB3 ATG
rpoB9 TTC
B
Frequence
E.Coli
505 F F G S S Q L S Q F M D Q N N P L S E I T H K R R I S A L G P G G 537
399 F F G T S Q L S Q F M D Q N N P L S G L T H K R R L S A L G P G G 431
M.Leprae
Frequence

FIG. 11

```
ValProGlyAlaProAsnArgIleSerPheAlaLysLeuArgGluProLeuGluValPro
GTGCCCGGCGCGCCCAACCGAATTTCATTTGCCAAGCTCCGCGAACCGCTTGAGGTTCCG        60

GlyLeuLeuAspValGlnThrAspSerPheGluTrpLeuIleGlySerProCysTrpArg
GGGCTACTTGATGTGCAGACTGATTCATTTGAGTGGTTGATCGGATCGCCGTGCTGGCGT       120

AlaAlaAlaAlaSerArgGlyAspLeuLysProValGlyGlyLeuGluGluValLeuTyr
GCAGCGGCCGCAAGCCGCGGCGATCTCAAGCCGGTGGGTGGTCTCGAAGAGGTGCTCTAC       180

GluLeuSerProIleGluAspPheSerGlySerMetSerLeuSerPheSerAspProArg
GAGCTGTCGCCGATCGAGGATTTCTCCGGCTCAATGTCATTGTCTTTCTCCGATCCCCGT       240

PheAspGluValLysAlaProValGluGluCysLysAspLysAspMetThrTyrAlaAla
TTTGACGAAGTCAAGGCGCCCGTCGAAGAGTGCAAAGACAAGGACATGACGTACGCGGCC       300

ProLeuPheValThrAlaGluPheIleAsnAsnAsnThrGlyGluIleLysSerGlnThr
CCGCTGTTCGTCACGGCCGAGTTCATCAACAACAACACCGGGGAGATCAAGAGCCAGACG       360

ValPheMetGlyAspPheProMetMetThrGluLysGlyThrPheIleIleAsnGlyThr
GTGTTTATGGGCGACTTCCCTATGATGACTGAGAAGGGAACCTTCATCATCAACGGGACC       420

GluArgValValValSerGlnLeuValArgSerProGlyValTyrPheAspGluThrIle
GAGCGTGTCGTCGTTAGCCAGCTGGTGCGCTCCCCTGGAGTATACTTCGACGAGACGATC       480

AspLysSerThrGluLysThrLeuHisSerValLysValIleProSerArgGlyAlaTrp
GACAAGTCCACAGAAAAGACGCTGCATAGTGTCAAGGTGATTCCCAGCCGCGGTGCCTGG       540

LeuGluPheAspValAspLysArgAspThrValGlyValArgIleAspArgLysArgArg
TTGGAATTCGATGTCGATAAACGCGACACCGTCGGTGTCCGCATTGACCGGAAGCGCCGG       600

GlnProValThrValLeuLeuLysAlaLeuGlyTrpThrSerGluGlnIleThrGluArg
CAACCCGTCACGGTGCTTCTCAAAGCGCTAGGTTGGACCAGTGAGCAGATCACCGAGCGT       660

PheGlyPheSerGluIleMetArgSerThrLeuGluLysAspAsnThrValGlyThrAsp
TTCGGTTTCTCCGAGATCATGCGCTCGACGCTGGAGAAGGACAACACAGTTGGCACCGAC       720
```

FIGURE 12(1)

```
GluAlaLeuLeuAspIleTyrArgLysLeuArgProGlyGluProProThrLysGluSer
GAGGCGCTGCTAGACATCTATCGTAAGTTGCGCCCAGGTGAGCCGCCGACTAAGGAGTCC    780

AlaGlnThrLeuLeuGluAsnLeuPhePheLysGluLysArgTyrAspLeuAlaArgVal
GCGCAGACGCTGTTGGAGAACCTGTTCTTCAAGGAGAAACGCTACGACCTGGCCAGGGTT    840

GlyArgTyrLysValAsnLysLysLeuGlyLeuHisAlaGlyGluLeuIleThrSerSer
GGTCGTTACAAGGTCAACAAGAAGCTCGGGTTGCACGCCGGTGAGTTGATCACGTCGTCC    900

ThrLeuThrGluGluAspValValAlaThrIleGluTyrLeuValArgLeuHisGluGly
ACGCTGACCGAAGAGGATGTCGTCGCCACCATAGAGTACCTGGTTCGTCTGCATGAGGGT    960

GlnSerThrMetThrValProGlyGlyValGluValProValGluThrAspAspIleAsp
CAGTCGACAATGACTGTCCCAGGTGGGGTAGAAGTGCCAGTGGAAACTGACGATATCGAC   1020

HisPheGlyAsnArgArgLeuArgThrValGlyGluLeuIleGlnAsnGlnIleArgVal
CACTTCGGCAACCGCCGGCTGCGCACGGTCGGCGAATTGATCCAGAACCAGATCCGGGTC   1080

GlyMetSerArgMetGluArgValValArgGluArgMetThrThrGlnAspValGluAla
GGTATGTCGCGGATGGAGCGGGTGGTCCGGGAGCGGATGACCACCCAGGACGTCGAGGCG   1140

IleThrProGlnThrLeuIleAsnIleArgProValValAlaAlaIleLysGluPhePhe
ATCACGCCGCAGACGCTGATCAATATCCGTCCGGTGGTCGCCGCTATCAAGGAATTCTTC   1200

GlyThrSerGlnLeuSerGlnPheMetAspGlnAsnAsnProLeuSerGlyLeuThrHis
GGCACCAGCCAGCTGTCGCAGTTCATGGATCAGAACAACCCTCTGTCGGGCCTGACCCAC   1260

LysArgArgLeuSerAlaLeuGlyProGlyGlyLeuSerArgGluArgAlaGlyLeuGlu
AAGCGCCGGCTGTCGGCGCTGGGCCCGGGTGGTTTGTCGCGTGAGCGTGCCGGGCTAGAG   1320

ValArgAspValHisProSerHisTyrGlyArgMetCysProIleGluThrProGluGly
GTCCGTGACGTGCACCCTTCGCACTACGGCCGGATGTGCCCGATCGAGACTCCGGAGGGC   1380

ProAsnIleGlyLeuIleGlySerLeuSerValTyrAlaArgValAsnProPheGlyPhe
CCGAACATAGGTCTGATCGGTTCATTGTCGGTGTACGCGCGGGTCAACCCCTTCGGGTTC   1440
```

FIGURE 12(2)

```
IleGluThrProTyrArgLysValValAspGlyValValSerAspGluIleGluTyrLeu
ATCGAAACACCGTACCGCAAAGTGGTTGACGGTGTGGTCAGCGACGAGATCGAATACTTG   1500

ThrAlaAspGluGluAspArgHisValValAlaGlnAlaAsnSerProIleAspGluAla
ACCGCTGACGAGGAAGACCGCCATGTCGTGGCGCAGGCCAACTCGCCGATCGACGAGGCC   1560

GlyArgSerSerSerArgAlaCysTrpValArgArgLysAlaGlyGluValGluTyrVal
GGCCGTTCCTCGAGCCGCGCGTGTTGGGTGCGCCGCAAGGCGGGCGAGGTGGAGTACGTG   1620

AlaSerSerGluValAspTyrMetAspValSerProArgGlnMetValSerValAlaThr
GCCTCGTCCGAGGTGGATTACATGGATGTCTCGCCACGCCAGATGGTGTCGGTGGCCACA   1680

AlaMetIleProPheLeuGluHisAspAspAlaAsnArgAlaLeuMetGlyAlaAsnMet
GCGATGATTCCGTTCCTTGAGCACGACGACGCCAACCGTGCCCTGATGgGCgCTAACATG   1740

GlnArgGlnAlaValProLeuValArgSerGluArgProLeuValGlyThrGlyMetGlu
CAGcgCCAAGCGGTTCCGTTGGTGCGCAGCGAACGACCGTTGGTGGGTACCGGTATGGAG   1800

LeuArgAlaAlaIleAspAlaGlyHisValValValAlaGluLysSerGlyValIleGlu
TTGCGCGCGGCCATCGACGCTGGCCACGTCGTCGTTGCGGAGAAGTCCGGGGTGATCGAG   1860

GluValSerAlaAspTyrIleThrValMetAlaAspAspGlyThrArgArgThrTyrArg
GAGGTTTCCGCCGACTACATCACCGTGATGGCCGATGACGGCACCCGGCGGACTTATCGG   1920

MetArgLysPheAlaArgSerAsnHisGlyThrCysAlaAsnGlnSerProIleValAsp
ATGCGTAAGTTCGCGCGCTCCAACCACGGCACCTGCGCCAACCAGTCCCCGATCGTGGAT   1980

AlaGlyAspArgValGluAlaGlyGlnValIleAlaAspGlyProCysThrGluAsnGly
GCGGGGGATCGGGTCGAGGCCGGCCAAGTGATTGCTGACGGTCCGTGCACTGAGAACGGC   2040

GluMetAlaLeuGlyLysAsnLeuLeuValAlaIleAsnAlaValGlyGlySerThrThr
GAGATGGCGTTGGGCAAGAACTTGCTGGTGGCGATCAATGCCGTGGGAGGGTCAACAACT   2100

AsnGluAspAlaIleIleLeuSerAsnArgLeuValGluGluAspValLeuThrSerIle
AACGAGGATGCGATCATCCTGTCTAACCGACTGGTCGAAGAGGACGTGCTTACTTCGATT   2160
```

FIGURE 12(3)

SUBSTITUTE SHEET

```
HisIleGluGluHisGluIleAspAlaArgAspThrLysLeuGlyAlaGluGluIleThr
CACATTGAGGAGCATGAGATCGACGCCCGTGACACCAAGCTGGGTGCTGAGGAGATCACC   2220

ArgAspIleProAsnValSerAspGluValLeuAlaAspLeuAspGluArgGlyIleVal
CGGGACATTCCCAACGTCTCCGATGAGGTGCTAGCCGACTTGGACGAGCGGGGCATCGTG   2280

ArgIleGlyAlaGluValArgAspGlyAspIleLeuValGlyLysValThrProLysGly
CGGATTGGCGCGGAGGTTCGTGACGGTGATATCCTGGTTGGCAAGGTCACCCCGAAGGGG   2340

GluThrGluLeuThrProGluGluArgLeuLeuArgAlaIlePheGlyGluLysAlaArg
GAAACTGAGCTGACACCGGAAGAGCGGTTGCTGCGGGCGATCTTCGGCGAAAAGGCCCGC   2400

GluValArgAspThrSerLeuLysValProHisGlyGluSerGlyLysValIleGlyIle
GAGGTCCGTGACACGTCGCTGAAGGTGCCACACGGCGAATCCGGCAAGGTGATCGGCATT   2460

ArgValPheSerHisGluAspAspAspGluLeuProAlaGlyValAsnGluLeuValArg
CGGGTGTTCTCCCATGAGGATGACGACGAGCTGCCCGCCGGCGTCAACGAGCTGGTCCGT   2520

ValTyrValAlaGlnLysArgLysIleSerAspGlyAspLysLeuAlaGlyArgHisGly
GTCTACGTAGCCCAGAAGCGCAAGATCTCTGACGGTGACAAGCTGGCTGGCGGCACGGC   2580

AsnLysGlyValIleGlyLysIleLeuProAlaGluAspMetProPheLeuProAspGly
AACAAGGGCGTGATCGGCAAGATCCTGCCTGCCGAGGATATGCCGTTTCTGCCAGACGGC   2640

ThrProValAspIleIleLeuAsnThrHisGlyValProArgArgMetAsnValGlyGln
ACCCCGGTGGACATCATCCTCAACACTCACGGGGTGCCGCGGCGGATGAACGTCGGTCAG   2700

IleLeuGluThrHisLeuGlyTrpValAlaLysSerGlyTrpLysIleAspValAlaGly
ATCTTGGAAACCCACCTTGGGTGGGTAGCCAAGTCCGGCTGGAAGATCGACGTGGCCGGC   2760

GlyIleProAspTrpAlaValAsnLeuProGluGluLeuLeuHisAlaAlaProAsnGln
GGTATACCGGATTGGGCGGTCAACTTGCCTGAGGAGTTGTTGCACGCTGCGCCCAACCAG   2820

IleValSerThrProValPheAspGlyAlaLysGluGluGluLeuGlnGlyLeuLeuSer
ATCGTGTCGACCCCGGTGTTCGACGGCGCCAAGGAAGAGGAACTACAGGGCCTGTTGTCC   2880
```

FIGURE 12(4)

SUBSTITUTE SHEET

```
SerThrLeuProAsnArgAspGlyAspValMetValGlyGlyAspGlyLysAlaValLeu
TCCACGTTGCCCAACCGCGACGGCGATGTGATGGTGGGCGGCGACGGCAAGGCGGTGCTC     2940

PheAspGlyArgSerGlyGluProPheProTyrProValThrValGlyTyrMetTyrIle
TTCGATGGGCGCAGCGGTGAGCCGTTCCCTTATCCGGTGACGGTTGGCTACATGTACATC     3000

MetLysLeuHisHisLeuValAspAspLysIleHisAlaArgSerThrGlyProTyrSer
ATGAAGCTGCACCACTTGGTGGACGACAAGATCCACGCCCGCTCCACCGGCCCGTACTCG     3060

MetIleThrGlnGlnProLeuGlyGlyLysAlaGlnPheGlyGlyGlnArgPheGlyGlu
ATGATTACCCAGCAGCCGTTGGGTGGTAAGGCACAGTTCGGTGGCCAGCGATTCGGTGAG     3120

MetGluCysTrpAlaMetGlnAlaTyrGlyAlaAlaTyrThrLeuGlnGluLeuLeuThr
ATGGAGTGCTGGGCCATGCAGGCCTACGGTGCGGCCTACACGCTGCAGGAGCTGTTGACC     3180

IleLysSerAspAspThrValGlyArgValLysValTyrGluAlaIleValLysGlyGlu
ATCAAGTCCGACGACACCGTCGGTCGGGTCAAGGTTTACGAGGCTATCGTTAAGGGTGAG     3240

AsnIleProGluProGlyIleProGluSerPheLysValLeuLeuLysGluLeuGlnSer
AACATCCCCGAGCCGGGCATCCCCGAGTCGTTCAAGGTGCTGCTCAAGGAGTTACAGTCG     3300

LeuCysLeuAsnValGluValLeuSerSerAspGlyAlaAlaIleGluLeuArgGluGly
CTGTGTCTCAACGTCGAGGTGCTGTCGTCCGACGGTGCGGCGATCGAGTTGCGCGAAGGT     3360

GluAspGluAspLeuGluArgAlaAlaAlaAsnLeuGlyIleAsnLeuSerArgAsnGlu
GAGGATGAGGACCTCGAGCGGGCTGCGGCCAACCTCGGTATCAACTTGTCCCGCAACGAA     3420

SerAlaSerIleGluAspLeuAla***
TCGGCGTCCATAGAAGATCTGGCTTAG     3447
```

FIGURE 12(5)

```
GlyAsnArgArgLeuArgThrValGlyGluLeuIleGlnAsnGlnIleArgValGlyMet
GGCAACCGCCGCCTGCGTACGGTCGGCGAGCTGATCCAAAACCAGATCCGGGTCGGCATG      60

SerArgMetGluArgValValArgGluArgMetThrThrGlnAspValGluAlaIleThr
TCGCGGATGGAGCGGGTGGTCCGGGAGCGGATGACCACCCAGGACGTGGAGGCGATCACA     120

ProGlnThrLeuIleAsnIleArgProValValAlaAlaIleLysGluPhePheGlyThr
CCGCAGACGTTGATCAACATCCGGCCGGTGGTCGCCGCGATCAAGGAGTTCTTCGGCACC     180

SerGlnLeuSerGlnPheMetAspGlnAsnAsnProLeuSerGlyLeuThrHisLysArg
AGCCAGCTGAGCCAATTCATGGACCAGAACAACCCGCTGTCGGGGTTGACGCACAAGCGC     240

ArgLeuSerAlaLeuGlyProGlyGlyLeuSerArgGluArgAlaGlyLeuGluValArg
CGACTGTCGGCGCTGGGGCCCGGCGGTCTGTCACGTGAGCGTGCCGGGCTGGAGGTCCGC     300

AspValHisProSerHisTyrGlyArgMetCysProIleGluThrProGluGlyProAsn
GACGTGCACCCGTCGCACTACGGCCGGATGTGCCCGATCGAAACCCCTGAGGGGCCCAAC     360

IleGlyLeuIleGlySerLeuSerValTyrAlaArgValAsnProPheGlyPheIleGlu
ATCGGTCTGATCGGCTCGCTGTCGGTGTACGCGCGGGTCAACCCGTTCGGGTTCATCGAA     420

ThrProTyrArg
ACGCCGTACCGC      432
```

FIGURE 13

FIGURE 14(1)

```
atgcccgatcacagggcactgcggcagggaataattgcactacgccaacatgttaacaac   20752

                                1
                            M  P  T  I  Q  Q  L  V  R  K  G
gaacacaatttacctgggagccggtatatgcccaccattcagcagctggtacgcaagggt   20812
                           _______________________c---------
                           ML51

 R  R  D  K  I  G  K  V  K  T  A  A  L  K  G  N  P  Q  R  R
cgtcgagacaagattggcaaggtcaagactgcggctctgaagggcaacccacagcgtcgc   20872
-----g--------ca-t-----------c----------------g---g--------t
                S                             S

 G  V  C  T  R  V  Y  T  S  T  P  K  K  P  N  S  A  L  R  K
ggtgtttgcacccgtgtgtacacttccaccccgaagaagccgaactcggcgcttcgcaag   20932
-----a--------c--------ca----t--------------------------g---
                         T

 V  A  R  V  K  L  T  S  Q  V  E  V  T  A  Y  I  P  G  E  G
gttgcccgcgtgaagctgacgagtcaggttgaggtcacagcgtacataccaggcgagggt   20992
--------------t--------------c--------g--------t--c-------cg
                                                          A

 H  N  L  Q  E  H  S  M  V  L  V  R  G  G  R  V  K  D  L  P
cacaacctacaggaacactccatggtgttggtgcgtggtggccgggtgaaagatctgcct   21052
--------g-----g-----g-----c--------c--c-----------g--c------
```

```
 G  V  R  Y  K  I  I  R  G  S  L  D  T  Q  G  V  K  N  R  K
ggtgtgcgttacaaaatcattcgcggttcgctcgacacccagggtgtcaagaaccggaag   21112
--------c-----g____________________________
                                           ML52

 Q  A  R  S  R  Y  G  A  K  K  E  K  S  *
caggctcgtagccgctatggagccaagaaggagaagagctga   21154
```

FIGURE 14(2)

```
                                    10
 R  K  G    R  R  D  K    I  G  K    V  K  T    A  A  L  K
CGCAAGGGTC GTCGAGACAA GATTGGCAAG GTCAAGACCG CGGCTCTGAA
   10             15                20                25

  G    N P    Q  R  R    G  V  C    T R    V  Y    T  S T
GGGCAGCCCG CAGCGTCGTG GTGTATGCAC CCGCGTGTAC ACCACCACTC
              30            35                40
  42
P  K  K  P    N   S A    L  R  K    V  A  R    V  K L  T
CGAAGAAGCC GAACTCGGCG CTTCGGAAGG TTGCCCGCGT GAAGTTGACG
        45                50             55

  S  Q  V    E  V  T  A    Y  I  P    G  E  G    H N  L  Q
 AGTCAGGTCG AGGTCACGGC GTACATTCCC GGCGAGGCGC ACAACCTGCA
    60              65                70                75

  E  H  S    M  V  L    V  R  G    G  R  V  K    D  L  P
GGAGCACTCG ATGGTGCTGG TGCGCGGCGG CCGGGTGAAG GACCTGCCTG
              80              85                90

 G  V  R  Y  K
 GTGTGCGCTAC AAG.
        95
```

FIG. 15

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 93/01063

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]	
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 C12Q1/68; //(C12Q1/68,C12R1:32)	

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	JOURNAL OF MOLECULAR BIOLOGY vol. 202, no. 1, August 1988, LONDON pages 45 - 58 JIN ET AL. 'Mapping and sequencing of mutations in the Escherichia coli rpoB gene that lead to rifampicin resistance' see the whole document	1,16,17,19
A		18,20
Y	WO,A,9 106 674 (SCOTGEN LTD) 16 May 1991 see page 3 - page 5	1,16,17,20
A		3-8,18,19
Y	EP,A,0 223 156 (HOECHST JAPAN LIMITED) 27 May 1987 see page 7 - page 10	1,16,17,20

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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"&" document member of the same patent family

3

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 AUGUST 1993	14.09.93
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	CEDER O.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	THE JOURNAL OF GENERAL MICROBIOLOGY vol. 60, no. 1, January 1970, LONDON pages 125 - 132 SRIPRAKASH ET AL. 'Isoniazid-resistant mutants of Mycobycterium tuberculosis H37RV: ...' cited in the application see abstract	2
P,X	NATURE vol. 358, no. 6387, 13 August 1992, LONDON pages 591 - 593 ZHANG ET AL. 'The catalase-peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis' cited in the application see the whole document	1-18
P,X	THE LANCET vol. 341, no. 8846, 13 March 1993, LONDON pages 647 - 650 TELENTI ET AL. 'Detection of rifampicin-resistance mutations in mycobacterium tuberculosis' see the whole document	1,16,17, 19
P,A	RESEARCH IN MICROBIOLOGY vol. 143, no. 7, September 1992, AMSTERDAM pages 721 - 730 HEYM ET AL. 'Isolation and characterization of isoniazid-resistant mutants of Mycobacterium smegmatis and M. aurum' cited in the application	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9301063
SA 74177

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 20/08/93
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9106674	16-05-91	None	
EP-A-0223156	27-05-87	AU-B- 587655	24-08-89
		AU-A- 6504286	14-05-87
		JP-A- 62201584	05-09-87

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